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BIOCHEMICAL CHARACTERISTICS OF ALDEHYDE AND LUCIFERASE MUTANTS OF LUMINOUS BACTERIA

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Communicated by B. H. Willier, December 15, 1954

Numerous studies on luminous bacteria have indicated that the light-emitting oxidative reaction is closely associated with the main electron-transport system of the cell. Investigations on cell-free extracts during the last two years have supported this general assumption. It is now clear that bacterial luciferase is a flavoprotein which catalyzes a light-emitting reaction in the presence of reduced flavin mononucleotide ($FMNH_2$) and a long-chain fatty aldehyde.[1, 2, 3, 4] No other cofactors have been found to be essential for this reaction. Light production by reduced diphosphopyridine nucleotide (DPNH) is due to the fact that this substance rapidly reduces riboflavin phosphate (FMN) in the presence of bacterial luciferase.[4, 5] In this light-emitting reaction, both reduced FMN and the long-chain aldehyde are consumed. On the basis of this and other recent observations we believe that the aldehyde oxidation or peroxidation by a flavin radical is the primary source of energy for luminescence.[5]

Numerous factors, such as salt concentration, oxygen pressure, and carbon source, have been found to influence bacterial luminescence in vivo; however, none of these factors has been entirely specific for light emission.[6, 7] Presumably these nonspecific effects are due to the fact that the luminescent system is dependent on other reactions essential to the viability of the organism. Recently, however, we have isolated a number of "dark" mutants of luminous bacteria, the growth requirements of which have not been altered. Light emission in two of these strains could be restored to normal by adding a long-chain aldehyde to the resting cells in the presence of glycerol. The other dark strains are incapable of light emission even in the presence of all of the cofactors known to be essential for luminescence. The present results imply that light emission in the cell is dependent on at least two enzyme systems, neither of which is required for growth of the organism. The product of one of these enzyme-catalyzed reactions is a long-chain aldehyde. The results also demonstrate that luciferase can be synthesized in luminous bacterial cells even though no light is emitted during growth.

Isolation of Mutants.—The salt-water bacterium *Achromobacter fischeri* was grown on the minimal liquid medium described by Farghaly,[8] to which 1 per cent peptone was added. An 18-hour culture was irradiated with ultraviolet light from a Westinghouse sterilamp, following the procedure described by McElroy and

Farghaly.[6] Biochemical mutant colonies were separated from the wild-type colonies by the replica plating method described by Lederberg and Lederberg.[9] The nonluminous mutants were detected on the nutrient agar plates by observing the individual colonies in the dark. The "dark" mutants grow as well as the normal wild type on both the complete and the minimal liquid medium, thus demonstrating no defect in growth accompanying the loss of luminescence.

Characteristics of the "Dark" Mutants.—Initial observations on suspensions of two of the dark mutants in the photomultiplier apparatus[10] revealed a very low but measurable luminescence from the living cells. This light intensity was not observable to the naked eye. The addition of numerous cofactors such as reduced pyridine nucleotides and flavins failed to stimulate light emission of these suspensions. However, the addition of 0.5 ml. of a saturated aqueous solution of dodecyl aldehyde to a 10-ml. suspension of these mutants stimulated light emission within five seconds. The addition of a similar quantity of aldehyde to the wild-type cells did not stimulate light emission. Thus far, two such mutants have been obtained which respond to the long-chain aldehydes; in addition, five other dark mutants have been isolated, the luminescence of which cannot be restored by any known factors. They have been classified as dark mutants of luminous bacteria on the basis of biochemical, nutritional, and morphological evidence.

Effect of Different Aldehydes on Luminescence.—Cormier and Strehler[1] reported that light emission in extracts of bacterial cells can be stimulated by a variety of long-chain aldehydes (from C_7 to C_{16}). We have made similar observations using cell-free extracts as well as the intact cells of the "dark" strains. The results with the intact cells agree perfectly with those obtained with the extracts. The results presented in Figure 1 show the time course for light emission in the intact bacterial mutants for two different aldehydes. In the isolated system maximum light emission is observed in less than one second when the reaction is initiated by adding aldehyde. It seems likely, therefore, that the rate of penetration of the aldehyde from the exterior must be the limiting factor.

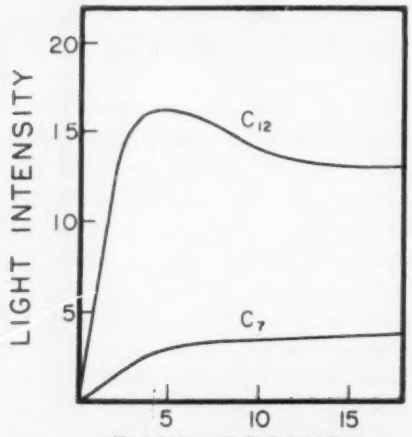

FIG. 1.—Light emission from luminous bacterial mutants upon the addition of aldehyde. C_{12} is the 12-carbon fatty aldehyde, and C_7 is the 7-carbon fatty aldehyde.

Isolation of Luciferase from the Dark Mutants.—Since light emission in some of the dark mutants occurs immediately upon the addition of an aldehyde, it is evident that luciferase and $FMNH_2$ are present. The presence of the luciferase has been confirmed by isolation and partial purification. Cells of the wild type and of dark mutants were grown in the peptone medium, and the luciferase was prepared as described by McElroy *et al.*[2] The amount of light emitted from various fractions in the presence of dodecyl aldehyde, DPNH, and FMN is shown in Table 1. It is evident that the dark strains which respond to aldehyde have a higher luciferase

content. On the other hand, none of the fractions from the other dark strains yielded light in the presence of aldehyde, DPNH, and FMN. Since part of the reaction sequence in the bacterial luciferase system is the reduction of FMN by $DPNH_2$, we tested the extracts from the dark mutants which failed to respond to aldehyde with chemically reduced FMN. None of the preparations emitted light. The possible presence of diffusible inhibitors in the extracts of the dark mutants was tested by mixing them with extracts of wild-type cells. No inhibition of light emission was observed under these conditions. We conclude, therefore, that the lack of luminescence in these dark mutants is not due to the presence of an inhibitor.

TABLE 1

PURIFICATION OF LUCIFERASE FROM MUTANT AND WILD TYPE*

Fraction	Wild-Type Strain	"Dark" Strain
Acid precipitate	44	1.7
$(NH_4)_2SO_4$ fractions		
0–30 per cent saturation	3.8	1.65
30–50 per cent saturation	60	228
50–65 per cent saturation	233	977

* The activities of the various fractions from the wild-type and the aldehyde-requiring luminous bacteria are expressed as light units per milligram of protein.

We have previously demonstrated that the natural aldehyde in luminous bacteria is bound to the protein (probably a lipoprotein) but can be liberated by ultraviolet irradiation.[2] Protein samples from the aldehyde-requiring dark strains have been irradiated, and the results indicate that aldehyde is released from these preparations. The evidence suggests that in these dark mutants the enzyme system involved in the freeing of an aldehyde has been blocked.

Discussion.—The restoration of normal light production in dark strains of luminous bacteria by the addition of a long-chain fatty aldehyde strongly suggests that the production of this substrate in the normal cell is under enzymatic control. Unfortunately, neither the nature of the naturally occurring "aldehyde" nor its original source has been identified, although apparently it can be liberated from bound structures by ultraviolet irradiation.[2] Since the depression of light emission by this mutation does not lead to more exacting growth requirements, it seems likely that the enzyme which has been affected is one concerned with the removal and transfer of an aldehyde from some lipid structure to luciferase. Because of the toxic effects of aldehyde and its apparent absence in crude extracts, it is doubtful whether free aldehyde is ever present in the concentration required to give normal luminescence. Attempts are now being made to isolate the natural substrate and the enzyme which makes it available for light emission.

The five other dark mutants apparently block the synthesis of the flavoprotein, luciferase. Efforts have been made to cross these dark strains, but unfortunately no luminous colonies have been observed. It is interesting that even in the absence of luminescence, i.e., absence of aldehyde, bacterial luciferase is still produced in normal amounts. It seems clear from other studies[3] that bacterial luciferase normally functions in electron transport without luminescence. The nonessentiality of this pathway is evident, however, from the fact that some of the viable dark cells do not contain the enzyme.

The present results suggest that light emission in luminous bacteria is under the control of at least two genetic factors which, as far as one can tell, do not affect other reactions essential for the growth of the organism. Bacterial luciferase is a flavoprotein which can catalyze the rapid oxidation of reduced pyridine nucleotides by molecular oxygen,[2] and it may have figured more prominently in the metabolism of primitive forms of luminous bacteria as well as other organisms. From an evolutionary standpoint it suggests the possibility that these biochemical reactions which support light emission may have had, in the past, some selective advantage for the survival of the organisms.

* This work was supported in part by the Office of Naval Research.

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*INTERACTIONS AMONG VARIANT AND WILD-TYPE STRAINS OF CELLULAR SLIME MOLDS ACROSS THIN AGAR MEMBRANES**

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Communicated by Kenneth B. Raper, November 8, 1954

The cellular slime molds are members of the phylum Myxomycophyta and the order Acrasiales. They display a complex developmental cycle[1] beginning with the germination of spores into phagotrophic myxamoebae. Following a period of exponential growth, the individual cells stream radially toward centers of aggregation under the influence of special "initiator" cells[2, 3] and in response to the production of specific chemical agents.[4, 5] The aggregates are then transformed into organized multicellular structures called "pseudoplasmodia." Each pseudoplasmodium undergoes additional morphogenetic alterations, ultimately producing, in the genus *Dictyostelium*, a fruiting structure with a mass of spores at the top, a parenchymatous, cellulose ensheathed stalk below, and in one species a basal disk.

These developmental changes are extremely orderly and undoubtedly subject to genetic control. Irradiation of the spores and myxamoebae of three species with ultraviolet light has enabled the isolation of many stable variant strains displaying aberrant development.[6] Among them is a class of morphogenetically deficient stocks which grow normally but cannot complete (and some cannot start) the morpho-

genetic sequence. Thus a number are aggregateless and remain as randomly dispersed myxamoebae after growth. Others (fruitless) can accomplish a part or the whole of the aggregation process, but all stop development short of the appearance of mature fruits.

It has been found[7] that cell mixtures of many pairs of deficient strains can develop synergistically to produce complete fruiting bodies with viable spores, although neither can do so alone. This synergism could be explained on at least four bases: (1) syngamy or heterokaryosis between the deficient partners to produce morphogenetically competent cells; (2) exchange of diffusible metabolites between the partners so as to shunt the biochemical blocks responsible for the deficiencies; (3) exchange of nondiffusible agents by direct cell contact; (4) need for the complete array of cell types necessary to normal development. Together, the deficient partners can provide this assembly, but neither can do so alone.

Serious doubt has been cast upon the first interpretation by the findings that (*a*) clonal platings of spores from synergistic fruitings produced no recombinants but only the original deficient phenotypes and (*b*) cells taken from any intermediate stage of the synergistic development did not yield recombinant clones.[8, 9] Thus, if syngamy or heterokaryosis occurs at all, it must be extremely rare. The present communication describes the results of experiments designed to distinguish among the remaining interpretations. The deficient strains have been allowed to develop on either side of agar membranes of a thickness as little as 30 μ, or about two cell diameters. It has been found that stocks which respond synergistically when mixed cannot counteract each other's developmental deficiencies if separated by a membrane. This result is made especially meaningful by the fact that the chemotactic substances responsible for aggregation, which have been shown to be exceedingly labile, can pass through membranes as thick as 200 μ. It would appear, therefore, that the assumption of synergism through exchange of diffusible intermediates is untenable at least for the collection of deficient stocks now at hand.

METHODS

A. ORGANISMS

Two species of slime molds were employed: *Dictyostelium mucoroides*, strain S-2, and *Dictyostelium discoideum*, strain NC-4. Of the former, only the wild type was used; of the latter, the wild type and a number of variant stocks to be specified later. The organisms were grown on glucose-peptone agar in association with *Aerobacter aerogenes*.[10]

B. PREPARATION OF CELLS FOR EXPERIMENT

After a stock had been grown for 48 hours at 22° C. and had reached the stationary growth phase, the myxamoebae were separated from the few remaining bacteria by differential centrifugation.[3] After three washes with cold distilled water, the cells were suspended in salt solution[4] and counted in a Levy chamber. Such suspensions can be dispensed on a washed agar–distilled water substratum so as to control both the number of cells and the population density.[2] Under these conditions the cells remain viable but do not increase in number. The wild-type populations aggregate and differentiate to produce normal fruitings with viable spores, while the variants attain their own distinctive levels of development.

C. PREPARATION OF AGAR MEMBRANES

Stainless-steel cylinders, machined from $^7/_8$-inch tubing, were employed in constructing the membranes. The cylinders have an outside diameter of 2.2 cm., a wall thickness of 1 mm., a length of 5 mm., and at one end an inside flange of 3 mm. Figure 1 shows the type of cylinder employed.

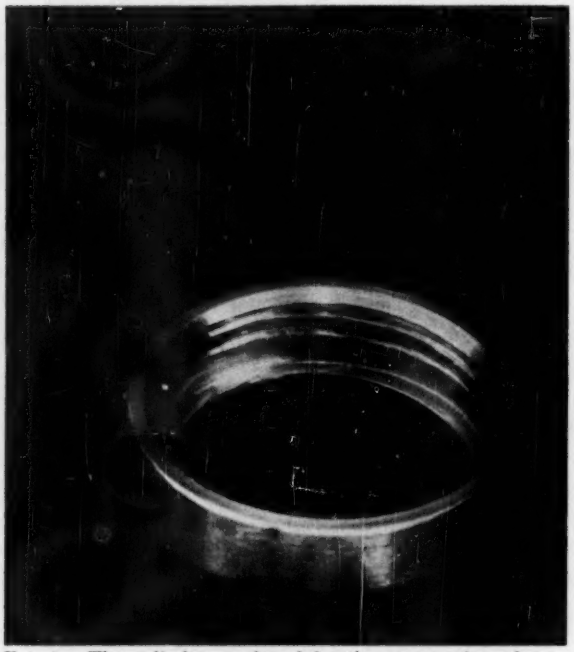

FIG. 1.—The cylinder employed for the preparation of agar membranes.

In order to make a membrane, a cylinder was held with forceps at the unflanged end and dipped into molten 2 per cent agar at 50°-60° C. When lifted out, a thin film of agar adhered to the flanged end. The assembly was then held perpendicularly for a time to allow the excess agar to drain away from the membrane, and this was removed by touching against the lip of a beaker. When the film had hardened, the assembly was placed, flanged end upward, in a paraffin imbedding jar over water-saturated filter paper. This jar was sealed with lanum and maintained at 22° overnight, after which the assembly was used for the experiments to be described.

A membrane made in this fashion is exceedingly thin. When thicker membranes (i.e., in excess of 100 μ) were required, a cylinder was placed flanged end downward on a glass slide. A measured volume of molten agar was pipetted within the flanged area. When the agar had hardened, the cylinder was pushed off the glass plate, the membrane adhering to the flange. The assembly was placed in an imbedding jar and treated as previously described.

D. MEASUREMENT OF MEMBRANE THICKNESS

Thickness determinations were performed with the aid of a microscope fine adjustment whose angular displacement had been calibrated in terms of the perpendicular displacement of the objectives. The distance through which the low-power objective had to travel to be focused first upon cells atop the membrane and then upon cells on the underside was taken to be the membrane thickness at that spot. The accuracy of the calibration was checked by measurement of a known distance (the height of the counting chamber in a Levy hemocytometer).

RESULTS

A. IMPREGNABILITY OF THE MEMBRANES

In order to test the membranes for the presence of holes through which organisms might pass, specimens were prepared from glucose-peptone agar under sterile conditions. An appropriately diluted suspension of *D. discoideum* spores, free of bacteria, was dispensed on one side of a membrane and a drop of *A. aerogenes* broth culture on the other. Such systems were incubated for as long as five days. Controls consisted of membranes where bacteria and spores had been inoculated on the same side. In addition, sterility controls to test for the presence of bacteria were run on the spore suspensions.

On control membranes, the growth was that to be expected from previous experience using agar plates. On the experimental membranes, the spores germinated, a process known to occur in the absence of bacteria; but growth of the myxamoebae was observed in only one membrane of the first ten tested, and this only after appreciable delay. It was surmised that air bubbles in the molten agar had produced this imperfection, and, when membranes with air bubbles were culled, no subsequent failures were encountered.

B. TESTS OF MORPHOGENETICALLY DEFICIENT STRAINS

In the original demonstration of synergistic development, washed cell suspensions of the deficient stocks in salt solution were mixed in paired combinations and in varying cell ratios and dispensed on a washed agar–distilled water substratum. Table 1 summarizes the types of synergistic structure observed. To test for exchange of diffusible materials, the stocks which had responded synergistically when paired as described above were washed and suspended in salt solution. Dilutions containing 10^6, 10^7, and 10^8 cells/cc. were prepared, and 0.01-cc. aliquots were placed

TABLE 1*

DEVELOPMENTAL BEHAVIOR OF THE DEFICIENT STRAINS, ALONE AND IN PAIRED COMBINATION

Strain	Extent of Development When Alone	Strain	Extent of Development When Alone	Extent of Synergistic Development
Fr-1	Loose aggregates	Fr-4	Pre-pseudoplasmodia	Immature fruits
		Agg-53	Dispersed myxamoebae	Mature, normal fruits
		Agg-53A	Dispersed myxamoebae	Mature, normal fruits
		Agg-59	Dispersed myxamoebae	Mature, normal, and thick-stemmed fruits
		Agg-204	Dispersed myxamoebae	Mature, normal, and bushy fruits†
Fr-2	Tightly knit but aberrant aggregates	Agg-59	Dispersed myxamoebae	Mature, normal, and bushy
		Agg-204	Dispersed myxamoebae	Mature, normal, and bushy fruits†
Fr-3	Pre-pseudoplasmodia	Agg-53	Dispersed myxamoebae	Mature, normal fruits
		Agg-53A	Dispersed myxamoebae	Mature, normal fruits
		Agg-59	Dispersed myxamoebae	Mature, normal fruits
		Agg-204	Dispersed myxamoebae	Mature, normal, and bushy fruits†
		Agg-206	Dispersed myxamoebae	Mature, normal fruits

* See text for experimental details. The term "mature" refers to the fact that the fruits possessed terminally developed spore and stalk cells. "Normal" indicates that the fruitings were indistinguishable from those of the wild type.

† "Bushy" refers to the appearance of a variant strain of NC-4 (Sussman and Sussman, *Ann. N.Y. Acad. Sci.*, **56**, 949, 1953). Agg-204 and Agg-206 were isolated from this stock.

on washed agar–distilled water membranes. After a few minutes the cells had lodged upon the agar surface, and the excess fluid could be drained away by decantation without disturbing the cells. Then the maneuver was repeated on the other side of each membrane with a suspension of myxamoebae from a second deficient strain. The synergistic pairs were tested in all possible cell ratios, as given above. In addition, all strains were tested against the wild type NC-4. The results can be categorized as follows:

1. *Wild Type versus Fruitless; Fruitless versus Fruitless.*—The wild type produced normal fruits; the fruitless variants, able to aggregate but incapable of further development, reached levels of development identical to those attained when the strains were incubated alone. The juxtaposition of another stock did not in any way affect the specific morphogenetic deficiencies.

2. *Wild Type versus Aggregateless; Fruitless versus Aggregateless.*—The wild type and fruitless strains developed precisely as they do when alone. The aggregateless cells were found to concentrate in small numbers directly above or below the centers of aggregation of the wild-type or fruitless forms.

3. *Fr-3 versus Any Other Strain.*—The fruitless stock Fr-3 differs from the other fruitless clones in that colonies on growth medium can proceed considerably beyond the aggregation stage. In contrast, if the cells are washed and placed on washed agar–distilled water medium, they aggregate but develop no further. When Fr-3 cells were dispensed on a washed agar membrane and opposed by any other strain, including aggregateless varieties, they were stimulated to equal the extent of development that they could achieve on growth medium. Thus, while passage of diffusible materials must have occurred in this instance, the result is rendered trivial by the nonspecificity of the response and the fact that the development was no more complete than on the growth medium.

C. THE PASSAGE OF HIGHLY LABILE CHEMOTACTIC AGENTS ACROSS THE MEMBRANE

The failure of the deficient stocks to develop synergistically when separated by a membrane is not of itself a crucial datum, since one might argue that the substances passed are extremely labile and are destroyed before penetrating the barrier. No answer to this objection can be conclusive, because, while there is a practical limit to how thin one can make the barrier, there is no limit to how labile one may imagine the substances to be. One can only hope to use as thin a membrane as possible and to demonstrate that agents known to be exceedingly labile can traverse the required distance. In order to satisfy the preceding requirement, a study was made of the ability of the chemotactic agent or agents associated with aggregation to pass through the membranes. Recently Shaffer[5] has succeeded in obtaining active preparations of the aggregative principles by leaching them from *D. discoideum* and *Polysphondylium violaceum* pseudoplasmodia. His results indicate that the half-lives of these agents at room temperature are of the order of 1 minute.

The agents, given the generic name "acrasin" by Bonner,[4] are produced by cells at the aggregative center and along the cell streams which converge upon it. Runyon[11] by covering myxamoebae spread on agar with a Visking sausage casing and placing other myxamoebae on top, was able to demonstrate the passage of a diffusible agent through the membrane, since the aggregative centers above and below coincided exactly. The same phenomenon has been explored in greater

detail by the writers, using agar membranes of different thicknesses. Washed wild-type myxamoebae at a concentration of 10^7 cells/cc. of salt solution were dispensed in 0.1-cc. aliquots on both sides of membranes of varied thicknesses. When aggregation was well under way, microscopic observation revealed degrees of attraction between aggregates above and below the membranes which could be categorized as follows: (1) *strong attraction:* the centers absolutely coincided, and the cell streams and branches thereof were precisely duplicated (see Fig. 2); (2) *weak attraction:* the centers coincided, but many of the streams and branches were not duplicated or, at best, were only approximately imitated above and below the membranes; (3) *no attraction:* the centers did not coincide, nor did the radially convergent streams.

When a pair of aggregates was found which could be indisputably relegated to one of the above categories, a determination was made of the membrane thickness at that point. Figure 3 summarizes the results obtained when myxamoebae of *D. discoideum* wild type opposed each other across the membranes and when similar

FIG. 2.—Strong chemotactic attraction between aggregates separated by a membrane. Cell streams atop the membrane are in focus. Coincident streams below the membrane are out of focus. ×200.

experiments were performed with *D. mucoroides* wild type. Regarding the first-named species, a strong aggregative attraction was observed at distances up to 140 μ, a weak response to 205 μ, and no attraction beyond 230 μ. The *D. mucoroides* system appears to be only slightly more sensitive. It was of interest to note that, when interspecific appositions were made between *D. discoideum* and *D. mucoroides,*

the coincidence of the heterologous aggregates followed the same pattern as shown in Figure 3, the sensitivity of response being no less than those of the homologous systems, a finding in agreement with the results obtained by Raper and Thom,[12] using cell mixtures of the two species.

The attractive potency of aggregates of deficient stocks when opposed by other deficient varieties or by the wild type was also observed. In general, the aggregates of any fruitless strain could be shown to coincide with those of any other fruitless or the wild type across thin membranes (30–100 μ). Attraction at greater distances

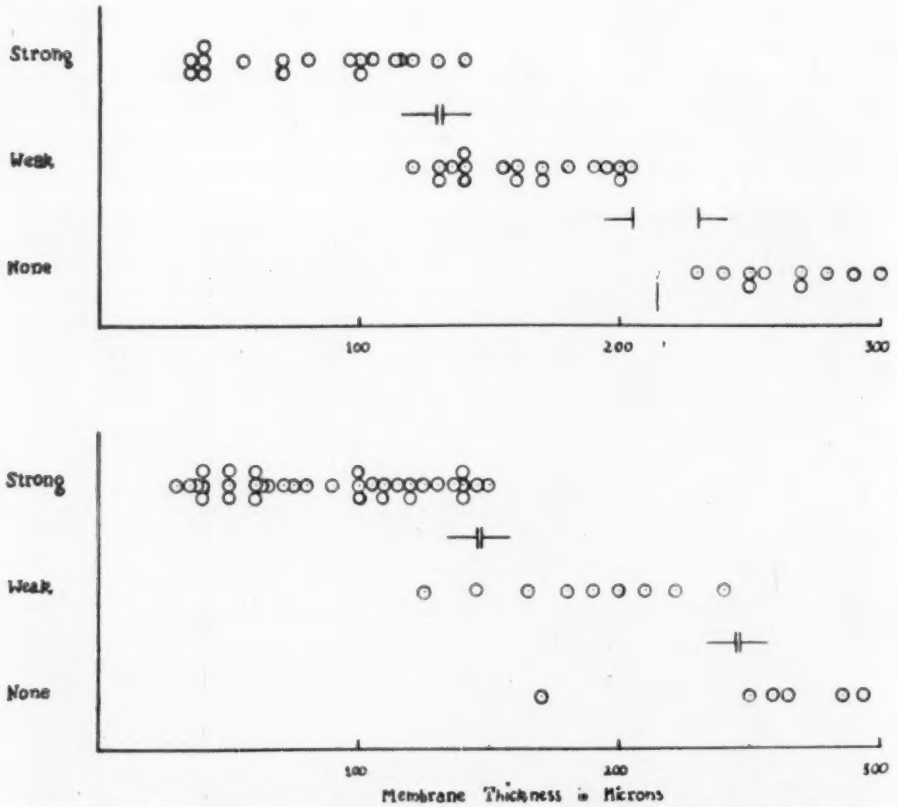

FIG. 3.—Chemotactic attraction across the membrane as a function of membrane thickness. Upper graph summarizes data obtained with wild-type myxamoebae of *D. discoideum;* lower, with *D. mucoroides.*

was not studied. The most interesting result was obtained when aggregateless myxamoebae were opposed by fruitless or wild-type cells across thin membranes. As mentioned previously, the aggregateless myxamoebae clustered directly above or below the opposed centers of aggregation. The areas within which the aggregateless cells were so affected were very small, being limited to a diameter of perhaps 0.2 mm. (to be compared with 3.0 mm., which is the approximate diameter of a wild-type aggregate, including the cell streams under the conditions employed). Subsequent study of more sensitive aggregateless stocks[13] has shown that some cluster not

only at the site of a wild-type center but, to a lesser extent, at the outlying streams as well. This is consistent with the results of Bonner,[4] which indicate that acrasin is produced both by the center and the streams. It has been shown[3] that the aggregateless strains can respond to the aggregative stimulus imposed when mixed with wild-type cells. It would seem, therefore, that while they may be sensitive to chemotactic agent, the aggregateless cells must be incapable of producing it, since the production of acrasin by large numbers of sensitized cells would inevitably be accompanied by the formation of ramified cell streams over extensive areas and presumably the concerted movement of these cells toward the center.

DISCUSSION

It has been shown that, while synergistic development cannot be accomplished by deficient stocks separated by as little as 30 μ of agar, at least one extremely labile, diffusible substance produced by the wild type can penetrate as much as 200 μ. It is the opinion of the writers that any interpretation of the synergistic morphogeneses based simply upon exchange of diffusible metabolites when considered in the light of these facts becomes highly improbable. One might argue that this is not the only requirement but that, in addition, specific geometric orientations must exist between the participating cells before effective exchanges can be accomplished. This extension of the argument at the level at which it must be applied seems no different from the assumption of exchange by direct cell contact. In any event, had diffusible substances any role in the synergistic process, it is reasonable to suppose that at least some interactions should have been observed, albeit with aberrant results.

Regarding the general applicability of the agar membrane system, it should be noted that the membranes are quite simple to prepare and manipulate. They might conceivably prove useful in many instances where interactions between cell populations must be observed on a solid substratum.

SUMMARY

Morphogenetically deficient variants of the slime mold *Dictyostelium discoideum* cannot complete the normal developmental sequence when alone but can do so synergistically when mixed in paired combinations. Such partners have been placed on opposite sides of agar membranes possessing thicknesses as little as 30 μ, or about two myxamoeboid cell diameters. With one exception, synergistic development could not be accomplished under these conditions. In contrast, a substance which has already been shown to be exchanged between the cells during development and is known to be both diffusible and highly labile could penetrate the membrane to a distance of at least 200 μ.

* This work was performed with the aid of funds supplied by the Office of Naval Research under contract NR 135-231 and by the National Institutes of Health, United States Public Health Service.

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*STRUCTURAL AND FUNCTIONAL ASPECTS OF THE A^b COMPLEXES IN MAIZE. 1. EVIDENCE FOR STRUCTURAL AND FUNCTIONAL VARIABILITY AMONG COMPLEXES OF DIFFERENT GEOGRAPHIC ORIGIN**

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Previous investigations[1] have established that the anomalous action of the A^b "allele" from Ecuador,[2] which is associated with purple plant and aleurone and dominant brown pericarp pigmentation, is ascribable to a gene complex of closely linked components which are separable by crossing-over. Thus the rare occurrences of the A^d or dilute-acting derivatives among gametes from A^b a plants are, in almost all cases, associated with crossing-over at the locus. Analyses of the A^d-bearing crossover strands in these experiments indicate that the A^d (designated α) component is the leftmost or proximal member of the complex. The dominant brown pericarp effect of A^b is attributable to this member of the complex. Certain technical difficulties have hindered the isolation and characterization of the rightmost component (designated β), but recent preliminary experiments indicate that it may be isolated on the reciprocal crossover strand. The β element, like A^b from which it derives, produces a purple plant and aleurone but differs from it in its determination of red pigment in the pericarp. Using the C notation for the centromere, the order of the members of the A^b complex is $C\alpha\beta$.

More recent studies[3, 4] indicate that most of the A^d-derivatives from A^b/A^b individuals also occur in association with crossing over. On the basis of this observation and additional evidence on the derivatives from certain special compounds involving A^b, it was concluded that α and β, or the segments in which they reside, are members of an adjacent duplication in which the genetic materials are ordered in the

same direction; i.e., A^b is a tandem, serial duplication. All the exceptional crossover derivatives which have been obtained from A^b may be explained on the basis of variable patterns of synapsis which are expected only if the members of the duplication have retained synaptic equivalence.

This paper, which is based on preliminary studies of a number of A^b alleles of Peruvian extraction, presents evidence for functional and structural variability among the A^b complexes of different origin, in respect to both the action of the individual members of the complex and the sequence of members within the duplication.

In 1945 a planting was made of seeds of diverse types from Peru (Bressman collection). The resulting plants were crossed, wherever possible, with *a a* tester plants in the hope of extracting various *A* alleles from the exotic plants. These studies not only identified several levels of effect among the extracted *A* alleles but indicated that there exist in these populations two different alleles with purple plant and aleurone effect—those associated with a red pericarp phenotype and those with a dominant brown pericarp effect. In several cases it was found that both of these alleles were carried in individual heterozygous plants of a Peruvian population. Because of its brown pericarp phenotype it was suspected that the latter type of allele is identical with the original A^b. Moreover, in several cases it was found that plants which are homozygous for an A^b allele of Peruvian origin give rise, with low frequency, to gametes which carry a mutant allele with a much lowered phenotypic effect in plant and aleurone tissue. While these derivatives produce an effect somewhat similar to that of the A^d, or dilute-acting allele, which originates from A^b (Ecuador), it has since become evident that they are consistently weaker in action and, indeed, are indistinguishable in effect from the allele designated a^p (pale) which was extracted by Emerson and Anderson[2] from a plant of Peruvian origin. At this point it seemed most reasonable to consider that these A^b alleles of different geographic origin are distinguishable only on the basis of the differing effects of their α components, even though evidence for the existence of a complex was available only for the A^b allele from Ecuador. In what follows, the α' notation will be employed in referring to this alpha element of lowered phenotypic effect.

In order to determine whether these A^b alleles of Peruvian extraction (the general designation A^b:P will be employed hereafter in referring to these as a group) represent complexes of the $\alpha'\beta$ type, several of these of separate origin were chosen for further investigation, and, in the cases of two of these alleles, designated A^b:Lima and A^b:Cusco, the experiments have been extensive. As expected, it was found that heterozygous A^b:P/*a* sh_2 (shrunken endosperm-2 is 0.25 crossover units to the right of *A*) individuals give rise infrequently to "mutants" having the characteristic pale phenotype. If the A^b:P alleles are complexes of the $\alpha'\beta$ type, with α' representing the leftmost element as in the case of the original A^b from Ecuador, it would be expected that crossovers which isolate the α' component should be recombinants for the *sh* locus and that the exceptional strands should have the constitution α' *sh*. Thus, in the case of similar heterozygotes involving the original A^b-allele, 92 per cent of all α derivatives are recombinants for the distal marker (in heterozygotes of this type, α itself serves as a proximal marker, and no additional marking to the left of the *A* locus is required). More than 180 pale (α') derivatives of independent occurrence have been obtained from A^b:P/*a sh* heterozygotes in experiments testing

well over 200,000 A^b:P gametes, and not one of these is a recombinant in the A^b-*Sh* region. This negative result is not due to some general suppression of crossing-over in the *A* region, since normal frequencies of purple-shrunken and colorless-nonshrunken crossovers are obtained in the same experiment. It may be concluded either that the A^b:P alleles involved are not complexes, in which case all the pale derivatives originate by some mechanism other than crossing-over, or that these alleles are complexes in which either (1) the arrangement of members is such as not to permit the isolation of the α' component by crossing-over or (2) the sequence of members within the duplication is reversed (i.e., $\beta\alpha'$), in which case, for lack of a proximal marker in the tested parents, an association between crossing-over and the occurrence of the pale derivatives could not possibly have been observed in the experiment described above.

To afford a more adequate test of these possibilities, both A^b:Lima and A^b:Cusco were made into compounds of the type A^b:P/*T a sh*, in which the heterozygous condition of the translocation 2–3d, which has its point of interchange in the long arm of chromosome 3 about six units from *A*, is employed as a proximal marker. The factor *et* (etched endosperm, virescent seedling; *ca.* 13 units distal to *A*) was substituted for *sh* in some of the experiments. The pale derivatives and their progenies from these sources were analyzed to determine the constitution of strands carrying the "mutant" pale factor. From these studies (Table 1) it is evident that, among the pale derivatives, noncrossover strands for the marked segments are considerably more frequent than crossovers.[5] Even so, the frequency of crossovers among the exceptional pale offspring (37 per cent) is far higher than would be expected if the origin of these derivatives were independent of crossing-over. Moreover, all the crossovers are of the type which is recombinant for the proximal marker. These findings indicate that A^b:Lima and A^b:Cusco are complexes in which the sequence of α' and β members is the reverse of that which is established for the original A^b allele (Fig. 1).

TABLE 1

CONSTITUTIONS OF 19 α'-BEARING STRANDS OCCURING AMONG THE OFFSPRING OF MARKED A^b:P/*a* INDIVIDUALS

SOURCE	A^b:P GAMETES TESTED	EXCEPTIONAL OFFSPRING: Noncrossovers (α')	EXCEPTIONAL OFFSPRING: Crossovers* ($T\alpha'$)
A^b:Lima/*T a sh*	6,900	9	3
A^b:Cusco/*T a sh*	5,200	1	3
A^b:Lima/*T a et*	1,300	2	1
A^b:Cusco/*T a et*	1,300	0	0
Totals	14,700	12	7

* Normal crossing-over for *T-sh* segment is *ca.* 6 per cent and for the *sh-et* interval *ca.* 13 per cent.

A crucial test of the foregoing hypothesis is afforded by an analysis of A^b/A^b:P heterozygotes. If, as supposed, the sequence of members differs in these two complexes, this heterozygote may be represented as $\alpha\beta/\beta\alpha'$. In this case the alpha-bearing crossover strands originating from appropriately marked heterozygotes should be restricted to those carrying only one of the two possible recombinant conditions for the marker genes. Table 2 summarizes the results of such an analysis, involving heterozygotes of A^b with A^b:Lima and A^b:Cusco and with another allele of Peruvian extraction, A^b:Arequipa. Prior analysis has established that this allele, like the other two, gives rise to an intermediate pale form and that this event is not

accompanied by recombination in the A^b-*Sh* segment. Of the fifteen exceptional alpha cases from these heterozygotes, three are noncrossovers for the *T*-*Sh* segment. The remaining twelve cases are all crossovers of one type, carrying the interchange and the normal allele of *et*. It is significant that the complementary crossover class, consisting of noninterchanged-*et* strands, is not represented among the α derivatives. The data therefore indicate not only that crossing-over is associated with the occurrence of the alpha derivatives but also that the sequence of alpha and beta members in the A^b:P complexes studied is the reverse of that in the A^b (Ecuador) complex (Fig. 2).

In view of the foregoing evidence, which indicates that the sequence of components in A^b is $\alpha\beta$, whereas that in the A^b:P complexes studied is $\beta\alpha'$, it might be considered that these complexes or duplications, as a whole, are inverted with respect to each other. However, the finding that wholly functional gametes carrying the alpha derivatives arise by crossing-over from A^b/A^b:P individuals is against this interpretation, since the latter requires that such crossovers occur within a small inversion loop, and under these circumstances, because of the resultant formation of dicentric bridges, the exceptional derivatives would not be included in functional gametes. Likewise, in heterozygotes with the same recessive *a*, both complexes yield, as a consequence of crossing-over, functional gametes carrying the alpha derivatives. The evidence indicates, therefore, that the one complex is not simply a gross inversion of the other but rather suggests that the members of the duplication have exchanged position while retaining the serial order of the duplication as a whole.

The special kind of evidence[3, 4] from homozygous A^b individuals and from various compounds involving this allele, which may be taken to indicate that the A^b complex is a serial duplication, is not available for the A^b:P complexes. However, from the results summarized in Table 2 and from a consideration of the scheme in Figure 2,

TABLE 2

CONSTITUTIONS OF ALPHA-BEARING STRANDS AMONG THE PROGENY OF MARKED A^b/A^b:P INDIVIDUALS

SOURCE	TOTAL GAMETES TESTED	EXCEPTIONAL OFFSPRING*			
		NONCROSSOVERS		CROSSOVERS†	
		T et	α	*T +*	*+ et*
T A^b *et*/A^b:Lima	7,500	1	0	4	0
T A^b *et*/A^b:Cusco	3,700	0	0	4	0
T A^b *et*/A^b:Arequipa	4,900	1	1	4	0
Total	16,100	2	1	12	0

* Two different levels of effect (not indicated here) have been established for the alpha derivatives from this source; see text for discussion.
† Normal recombination value for the *T*-*et* segment is *ca.* 18 per cent.

it is apparent that functional alpha derivatives would be expected among the progeny of A^b/A^b:P plants only if one or more members of the A^b:P duplication are ordered in the same direction as are those of A^b. Table 2 gives no indication of another interesting feature concerning the alpha derivatives from TA^bet/A^b:P heterozygotes. The noncrossover derivative carrying the *T* and *et* markers is, as expected, of the α, or deeper, type, whereas the other class of noncrossover is of the α', or lighter, type. This is expected, since these derivatives must originate from the A^b ($\alpha\beta$) and A^b:P($\beta\alpha'$) complexes, respectively. However, the alpha derivatives which arise by crossing-over (all are of one type, carrying *T* and the normal allele of

et) have different levels of effect. Not all of these have been subjected to critical comparisons in which the effects of modifiers may be excluded as a basis for differences, but, among those that have, at least two different levels of alpha deriva-

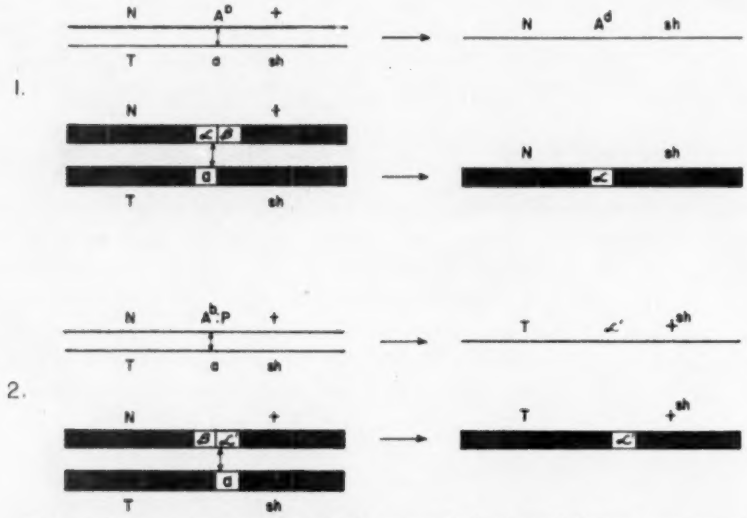

FIG. 1.—Schemes to account for the crossover isolation of (1) the α component of A^b (Ecuador), accompanied by recombination for the distal marker, and (2) the α' component of A^b:P (Peru), accompanied by recombination for the proximal marker; the schemes are based on the assumption of a different sequence of the α and β members in the two complexes.

FIG. 2.—Scheme to account for the isolation of the pale (alpha) derivatives on crossover strands of only one type from A^b/A^b:P heterozygotes, based on different sequence of members in the two complexes. See text for evidence on alternative patterns of synapsis and crossover sites.

tives have been distinguished, those which are similar to α and those which resemble α' in effect. To account for these results it is necessary to assume that the A^b and A^b:P complexes may engage in oblique synapsis of the type shown below:

T			*et*
	$\ldots\alpha\ldots$	$\ldots\beta\ldots$	
	$\ldots\beta\ldots$	$\ldots\alpha'\ldots$	
+			+

In this case a crossover in the rightmost region of the alpha-carrying segment would isolate the α component, while an exchange in the left region of the same segment would explain the occurrence of the α' derivative. In any case, the occurrence of this kind of oblique synapsis in heterozygotes may not be taken as critical evidence that members of the A^b:P duplication are ordered in serial fashion. For example, any one of the possible alpha derivatives, including the $\alpha\alpha'$ complex (Fig. 2), for which evidence is neither available nor likely to be obtained easily, may occur as a result of crossovers within the α' segment of A^b:P, and this would not necessarily require that the β segment of this complex have the same order. Critical evidence bearing on the serial nature of the A^b:Lima, A^b:Cusco, and A^b:Arequipa duplications must await the conclusion of experiments dealing with marked, homozygous A^b:P plants. However, it appears, from the arguments which follow, that this is the most likely arrangement.

We may now consider the possible evolutionary basis for the changed sequence of the α and β components in A^b as compared with the A^b:P complexes. One possible explanation is based on the assumption that, following the initial event giving rise to the duplication, isolated races have experienced similar though not identical mutational events in different members of the duplication. On this scheme the fundamentally similar action of the two alphas is a coincidence. This would appear to be the only reasonable explanation, were it not for the evidence indicating that the members of the A^b complex are homologous and may undergo oblique synapsis in the homozygote.[4] Crossing-over within the complex in this case renders the duplication unstable and may be expected to lead, on the one hand, to its loss and, on the other, to a higher order of replication. In the absence of a deleterious effect of the latter, certain combinations of heterozygotes in succeeding generations could, when accompanied by crossing-over, lead to a change in the original sequence of the members of the duplication. One such possible scheme to account for changes in sequence of members of the A^b complexes is illustrated in Figure 3. It is possible on this mechanism to account for changes in sequence, as well as in numbers, of members.

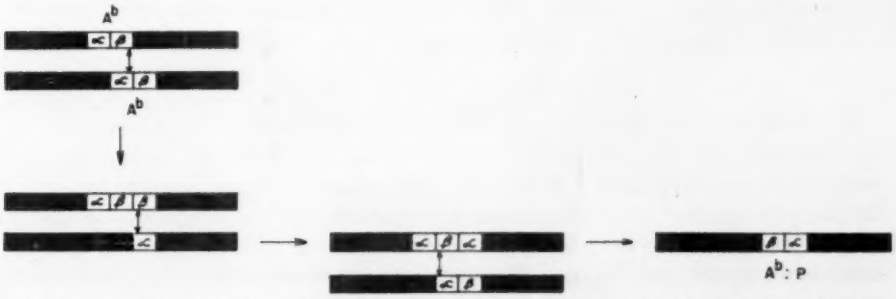

FIG. 3.—Hypothetical scheme to account for a change in sequence of A^b components based on crossing-over within obliquely synapsed members of the complex.

It should be noted that the bar-infrabar and infrabar-bar complexes of Sturtevant[6] represent instances of a somewhat similar change in sequence of members. For purposes of the present argument it is significant that adjacent, serial duplications were later established[7] as the basis for these changes. However, this finding poses a further question concerning the nature and site of the infrabar mutation.

It is proposed that the altered sequence of members of the A^b:P complexes is due to crossing-over within serial complexes whose members have retained synaptic equivalence rather than to parallel mutation in different members of the complex. However, it is apparent that neither mechanism would be expected to modify the serial order of members of the duplication.

Summary.—Analyses of several A^b alleles of Peruvian extraction indicate that they consist of complexes whose members are separable by crossing-over. These complexes differ from that of A^b, the original "allele" from Ecuador, in respect to both the level of effect of the α component and the sequence of α and β members in the complex. The changed sequence of members is not ascribable to gross inversion of the complex but is explained if it is assumed that the A^b complexes are adjacent, serial duplications whose members are homologous.

* Journal Series Paper No. 1482, approved by the director of the Missouri Agricultural Experiment Station. This is a report of research conducted in the Department of Botany, University of Illinois. The author wishes to express appreciation to the Pioneer Hi-Bred Corn Company for stocks of the original Bressman collection used in these studies.

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[5] These noncrossover derivatives which accompany those of crossover origin are found in progenies of the original A^b also, and the same situation is known in the case of certain other loci for which evidence of a complex is available. In a paper to appear elsewhere it is proposed that they occur at meiosis as a result of a crossover within a foldback involving paired members of the serially arranged complex.

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*SPLITTING OF VALUATIONS IN EXTENSIONS OF LOCAL DOMAINS**

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1. *Splitting of Valuations.*—Let R be a regular local domain of dimension >1 with maximal ideal M, such that the quotient field K of R and the residue field R/M have equal characteristic. Let K^* be a finite separable extension of K ($K \neq K^*$). In this note we shall prove the existence of infinitely many real discrete valuations of K with center M in R,[1] which split in K^*. This theorem probably holds under much more general conditions. The junior (first-named) author plans to study the generalization of this theorem in a subsequent note.

We shall consistently use the following notations: (1) If v is a valuation of a field K, then R_v will denote the valuation ring of v and M_v will denote the maximal ideal of R_v. (2) If p is any prime ideal in a domain R and if v is a valuation of the quotient field K of R, v is said to have center p in R if $R_v \supset R$ and $M_v \cap R = p$. (3) If R is a local ring and M its maximal ideal, we shall express this by saying, "(R, M) is a local ring."

LEMMA 1. *Let R be a Noetherian domain with quotient field K and let A be an ideal in R different from (0) and R. Then there exists a real discrete valuation v of K such that $R_v \supset R$ and $M_v \supset A$.*

Proof:[2] Let $\{u_1, u_2, \ldots, u_s\}$ be a basis of A. It is known that there exists a valuation w of K such that $R_w \supset R$ and $M_w \supset A$. We fix such a valuation w, and we label the u_i so as to have $w(u_1) \leq w(u_i)$ for $i = 2, 3, \ldots, s$. Let $R^1 = R[u_2/u_1, u_3/u_1, \ldots, u_s/u_1]$, whence $AR^1 = u_1R^1$. Since $M_w \supset A$, we have $w(u_1) > 0$, and u_1R^1 is not the unit ideal. Since R^1 is Noetherian, it follows that all the isolated primes of the principal ideal u_1R^1 are minimal. Let p be one of the isolated primes of u_1R^1. Let $S = R^1{}_p$, and let $m = pS$. Then S is a Noetherian domain with only one prime ideal different from (0) (i.e., S is a local domain of dimension 1). Therefore, the integral closure of S in K is the intersection of a finite number of valuation rings belonging to real discrete valuations of K.[3] Let v be one of these valuations. Then $R_v \supset S$ and $M_v \supset m$, and hence $R_v \supset R$ and $M_v \supset A$.

LEMMA 2. *Let (R, M) be an integrally closed local domain with quotient field K. Let u be an element of K such that neither u nor $1/u$ belongs to R. Then there exists a real discrete valuation v of K with center M in R such that $v(u) > 0$.*

Proof: Let $R_1 = R[u]$ and $M_1 = MR_1$. Then, clearly, $M_1 = M[u]$. Suppose, if possible, that $M_1 = R_1$, i.e., that $1 \in M_1$. Then there exist elements $a_0, a_1, \ldots, a_s$ in M such that $1 = a_0 + a_1u + \ldots + a_su^s$. Now $(a_0 - 1)$ is not in M and hence is a unit in R. Therefore, $b_i = a_i/(a_0 - 1) \in R$ for $i = 1, 2, \ldots, s$. Since $(1/u)^s + b_1(1/u)^{s-1} + b_2(1/u)^{s-2} + \ldots + b_s = 0$, and, since R is integrally closed, it follows that $1/u \in R$, a contradiction.

Therefore, $M_1 \neq R_1$. Hence, by Lemma 1, there exists a real discrete valuation v of K such that $R_v \supset R_1$ and $M_v \supset M_1$, and hence $R_v \supset R$, $R \cap M_v = M$, and $v(u) > 0$.

LEMMA 3. *Let (R, M) be a regular local domain of dimension $s > 1$ with quotient field K and residue field k. Let $\{x_1, x_2, \ldots, x_s\}$ be a set of regular parameters in R. Let $y_1 = x_1$, $y_i = x_i/x_1$ for $i = 2, 3, \ldots, s$; and let $S = R[y_2, y_3, \ldots, y_s]$. Then $y_1S \cap R = M$, and $k = R/M$ can be canonically identified with a subfield of $S/(y_1S)$. Furthermore, the residues $\bar{y}_2, \bar{y}_3, \ldots, \bar{y}_s$ modulo y_1S of $y_2, y_3, \ldots, y_s$ are algebraically independent over k, and $S/(y_1S)$ can be canonically identified with the polynomial ring $k[\bar{y}_2, \bar{y}_3, \ldots, \bar{y}_s]$ in $s - 1$ independent variables.*

Proof:[4] For any element u of R we set $v(u) =$ the leading degree of u; i.e., $v(u)$ is characterized by the property that $M^{v(u)}$ is the highest power of M which contains u. Furthermore, for $u_1, u_2 \in R$ we set $v(u_1/u_2) = v(u_1) - v(u_2)$. It follows from well-known properties of regular local rings that v is a real discrete valuation of K. We shall call this valuation v the *M-adic valuation* of R. Now $R_v \supset S$ and $v(y_1) > 0$, and hence $1 \notin y_1S$. Therefore, $y_1S \cap R \neq R$. Since $y_1S \supset M$ and since M is the maximal ideal in R, it follows that $y_1S \cap R = M$; and hence we can canonically identify $k = R/M$ with a subfield of $S/(y_1S)$, in the usual fashion. With this identification, it is clear that $S/(y_1S)$ is generated, as a ring over k, by $\bar{y}_2, \bar{y}_3, \ldots, \bar{y}_s$. Note that the center of v in R is the maximal ideal M, while the center of v in S is the minimal prime ideal y_1S. The valuation ring R_v is the quotient ring of S with respect to y_1S.

The only thing that remains to be shown is that $\bar{y}_2, \bar{y}_3, \ldots, \bar{y}_s$ are algebraically independent over k. Assume the contrary. Then there exists a polynomial

$f(y_2, y_3, \ldots, y_s) = \sum f_{i_2, i_3, \ldots, i_s} y_2^{i_2} y_3^{i_3} \ldots y_s^{i_s}$, with the coefficients $f_{i_2, i_3, \ldots, i_s}$ in R, but not all in M, such that $f(y_2, y_3, \ldots, y_s) \equiv 0 \pmod{y_1 S}$, i.e.,

$$f(y_2, y_3, \ldots, y_s) = y_1 g(y_2, y_3, \ldots, y_s),$$

where $g(y_2, y_3, \ldots, y_s)$ is a polynomial in $y_2, y_3, \ldots, y_s$ with coefficients in R. Multiplying both sides of the above equation by a suitable high power x_1^t of x_1 and setting

$$F_t(x_1, x_2, \ldots, x_s) = \sum f_{i_2, i_3, \ldots, i_s} x_1^{t-i_2-i_3-\ldots-i_s} x_2^{i_2} x_3^{i_3} \ldots x_s^{i_s},$$

we get

$$F_t(x_1, x_2, \ldots, x_s) = x_1^{t+1} g(y_2, y_3, \ldots, y_s).$$

Hence $v(F_t(x_1, x_2, \ldots, x_s)) \geq t + 1$, i.e., $F_t(x_1, x_2, \ldots, x_s)$ is contained in M^{t+1}. This is a contradiction, since $F_t(x_1, x_2, \ldots, x_s)$ is a form of degree t in $x_1, x_2, \ldots, x_s$ with coefficients in R, not all in M. Hence $\bar{y}_2, \bar{y}_3, \ldots, \bar{y}_s$ are algebraically independent over k, and this completes the proof.

COROLLARY 1. *Let m be any maximal ideal in S which contains y_1. Let $R^* = S_m$ and $m^* = mR^*$. Then R^* is a regular local ring of dimension s and y_1 is part of a minimal basis of m^* (whence y_1 belongs to a regular system of parameters of R^*).*

Since $m/(y_1 S)$ is a zero dimensional ideal in the polynomial ring $k[\bar{y}_2, \bar{y}_3, \ldots, \bar{y}_s]$, it follows by Lemma 9 of a previous paper[5] that $m/(y_1 S)$ has a basis $\{z_2, z_3, \ldots, z_s\}$ of $(s-1)$ elements. Therefore, m, and hence also m^*, has the basis $\{y_1, z_2, z_3, \ldots, z_s\}$ of s elements. Hence dim $R^* \leq s$. Since $m/(y_1 S)$ is of rank $s-1$, it follows that m^* is of rank $\geq s$. Therefore, dim $R^* = s$; hence R^* is regular, and y_1 is part of the minimal basis $\{z_1, z_2, \ldots, z_s\}$ of m^*, where $z_1 = y_1$.

Definition: We shall say that the ring R^* of Corollary 1 is obtained from R by a *quadratic transformation*. If we obtain a regular local ring $\bar{R}$ from R by applying n successive quadratic transformations, then we shall call $\bar{R}$ an nth quadratic transform of R.

COROLLARY 2. *Let $u_1, u_2, \ldots, u_h$ be a finite number of nonzero elements of M. Then there exist infinitely many maximal ideals m in S containing y_1 such that we have $u_i = y_1^{r_i}\delta_i$ for $i = 1, 2, \ldots, h$, where r_i is a positive integer and δ_i is a unit in $R^* = S_m$.*

Let r_i be the leading degree of u_i, and let $u_i = v_i(x_1, x_2, \ldots, x_s) + w_i$, where $v_i(X_1, X_2, \ldots, X_s)$ is a form of degree r_i in $R[X_1, X_2, \ldots, X_s]$ and $w_i \in M^{r_i+1}$. Let $a_i(\bar{y}_2, \bar{y}_3, \ldots, \bar{y}_s) = \bar{v}_i(1, \bar{y}_2, \bar{y}_3, \ldots, \bar{y}_s)$ where $\bar{v}_i(X_1, X_2, \ldots, X_s)$ is the form obtained from $v_i(X_1, X_2, \ldots, X_s)$ by reducing the coefficients modulo M. Let V_i be the hypersurface $a_i(\bar{y}_2, \bar{y}_3, \ldots, \bar{y}_s) = 0$ in the affine space A_{s-1}/k of the variables $\bar{y}_2, \bar{y}_3, \ldots, \bar{y}_s$. Now there are infinitely many points in A_{s-1} (rational or algebraic over k) which do not lie on any of the hypersurfaces $V_1, V_2, \ldots, V_h$, i.e., there exist infinitely many maximal ideals $\bar{m}$ in $k[\bar{y}_2, \bar{y}_3, \ldots, \bar{y}_s]$ which do not contain $a_i(\bar{y}_2, \bar{y}_3, \ldots, \bar{y}_s)$ for $i = 1, 2, \ldots, h$. Fix one such maximal ideal $\bar{m}$ in $k[\bar{y}_2, \bar{y}_3, \ldots, \bar{y}_s]$, and let m be the maximal ideal in S containing y_1 such that $m/(y_1 S) = \bar{m}$. Let $R^* = S_m$ and $m^* = mR$. Since $\bar{v}_i(1, \bar{y}_2, \bar{y}_3, \ldots, \bar{y}_s)$ is not in $\bar{m}$, it follows that $d_i = v_i(1, y_2, y_3, \ldots, y_s)$ is not in m and hence is a unit in R^*. Since $w_i \in M^{r_i+1} \subset y_1^{r_i+1} R^*$ and since $u_i = v_i(x_1, x_2, \ldots, x_s) + w_i = y_1^{r_i} d_i + w_i$, it follows that $u_i = y_1^{r_i}\delta_i$, where δ_i is a unit in R^*, as asserted. Also, the infinitely many choices of $\bar{m}$ give us infinitely many maximal ideals m in S with the required properties.

COROLLARY 3. *Let $h(X)$ be an irreducible nonconstant polynomial in $k[X]$. Then there exists a real discrete valuation v of K such that (a) v has center M in R and (b) $h(X)$ has a linear factor in $(R_v/M_v)[X]$.*

The ideal $\bar{m} = (h(\bar{y}_2), \bar{y}_3, \ldots, \bar{y}_s)$ in $k[\bar{y}_2, \bar{y}_3, \ldots, \bar{y}_s]$ is a maximal ideal. Let m be the maximal ideal in S containing y_1 such that $m/(y_1S) = \bar{m}$. Let $R^* = S_m$, and let $m^* = mR^*$. Then $h(X)$ has a linear factor in $(R^*/m^*)[X]$. It is obvious that any real discrete valuation v of K with center m^* in R^* has properties (a) and (b), and, by Lemma 1, there exist real discrete valuations v of K with center m^* in R^*.

After these lemmas let us restate and prove our main theorem.

THEOREM 1. *Let (R, M) be a regular local domain of dim $s > 1$, such that the quotient field K and the residue field k of R both have the same characteristic p. Let K^* be a finite separable algebraic field extension of K $(K \neq K^*)$. Then there exist infinitely many real discrete valuations v of K having the following two properties: (1) v has center M in R, and (2) v has more than one extension to K^*.*

Proof: Let α be a primitive element of K^*/K which is integral over R, and let

$$F(X) = X^n + f_1X^{n-1} + f_2X^{n-2} + \ldots + f_n \qquad (f_i \in R)$$

be the minimal monic polynomial of α over K. Let $\{x_1, x_2, \ldots, x_s\}$ be a minimal basis of M. Upon replacing, if necessary, α by αx_1, we may assume that $f_i \in M$ for $i = 1, 2, \ldots, n$.

Now we shall divide the argument into two cases.

Case 1, $p \neq 0$ *and* $n \equiv 0(p)$: Let $y_1 = x_1$, $y_i = x_i/x_1$ for $i = 2, 3, \ldots, s$; and let $S = R[y_2, y_3, \ldots, y_s]$. By Corollary 2 of Lemma 3, there exist infinitely many maximal ideals m in S such that, for those coefficients f_i which are different from zero, we have $f_i = y_i^{r_i}\delta_i$, $r_i > 0$, where δ_i is a unit in $R^* = S_m$. Let us fix one such m, and let $m^* = mR^*$. By Corollary 1 of Lemma 3, y_1 is part of a minimal basis $\{z_1, z_2, \ldots, z_s\}$ of m^*, where $z_1 = y_1$. Let $\beta = \alpha - z_2$. Then also β is a primitive element of K^*/K, and the minimal monic polynomial $G(X)$ of β over K is given by

$$G(X) = F(X + y_2) = X^n + g_1X^{n-1} + g_2X^{n-2} + \ldots + g_n \qquad (g_i \in R^*).$$

Since K^*/K is separable and $n \equiv 0(p)$, there exists at least one coefficient $f_i \neq 0$ such that $i \not\equiv 0(p)$. Let e be the smallest value of i such that $i \not\equiv 0(p)$ and $f_i \neq 0$. Then $g_e = f_e = z_1^{r_e}\delta_e$. Also $g_n = F(z_2) = z_2^n + Qz_1$ with $Q \in R^*$.

Fix an integer $q > n/e$, and let $u = g_ng_e^{-q}$. Since g_n is a nonunit which does not belong to the minimal prime ideal z_1R^*, it follows that $u \in R^*$ and that $g_e^q \in g_nR^*$, i.e., $1/u \in R^*$. By Lemma 2, there exists a real discrete valuation v of K with center m^* in R^* such that $v(u) \geq 0$. The valuation v then has center M in R. Also, $v(u) \geq 0$ implies that

$$v(g_n) \geq qv(g_e) > \left(\frac{n}{e}\right)v(g_e), \qquad \text{i.e.,} \qquad v(g_e) < \left(\frac{e}{n}\right)v(g_n).$$

Therefore, v has more than one extensions to K^*.[6] Since v has center m in S, it follows that distinct choices of the maximal ideal m in S give us distinct valuations v of the required type.

Case 2, $p = 0$, *or* $p \neq 0$ *and* $n \not\equiv 0(p)$: Upon replacing, if necessary, α by $\alpha - f_1/n$ we may assume that $f_1 = 0$. Let $y_1 = x_1$, $y_i = x_i/x_1$ for $i = 2, 3, \ldots, s$; and

let $S = R[y_2, y_3, \ldots, y_s]$. By Corollary 2 of Lemma 3, there exist infinitely many maximal ideals m in S such that for the nonzero coefficients f_i we have

$$f_i = y_1^{r_i}\,\delta_i \quad (r_i > 0), \tag{I}$$

where δ_i is a unit in $R^* = S_m$. Let us fix one such m, and let $m^* = mR^*$. By Corollary 1 of Lemma 3, y_1 is part of a minimal basis $\{z_1, z_2, \ldots, z_s\}$ of m^*, where $z_1 = y_1$. If for some i we have $r_i < (i/n)r_n$, then any real valuation of K with center m^* in R^* will split in K^*.[6] Now assume that

$$r_i \geq i/n \qquad \text{for all } i. \tag{II}$$

Let $t_1 = z_1 z_2^{-n} - d$, d a unit in R^*; $t_2 = z_2$; and $t_i = z_i z_2^{-n}$ for $i = 3, 4, \ldots, s$. Let $\mathfrak{S} = R^*[t_1, t_2, \ldots, t_s]$, $\mathfrak{m} = (t_1, t_2, \ldots, t_s)\mathfrak{S}$, $\mathfrak{S}^* = \mathfrak{S}_{\mathfrak{m}}$, and $\mathfrak{m}^* = \mathfrak{m}\mathfrak{S}^*$. Then it is easily seen that the ring $\mathfrak{S}^*$ is an nth quadratic transform of R^* and that it has $\{t_1, t_2, \ldots, t_s\}$ as a regular system of parameters. Let $\beta = \alpha t_2^{-r_n}$. Then also β is a primitive element of K^*/K, and its minimal monic polynomial $G(X)$ over K is given by

$$\begin{aligned} G(X) &= t_2^{-nr_n} F(t_2^{r_n} X) \\ &= t_2^{-nr_n}\,[t_2^{nr_n} X^n + z_1^{r_1}\delta_2 t_2^{r_n(n-2)} X^{n-2} + \ldots + z_1^{r_i}\delta_i t_2^{r_n(n-i)} X^{n-i} \\ &\qquad + \ldots + z_1^{r_n}\delta_n] \qquad \text{by (I)} \\ &= X^n + g_2 X^{n-2} + g_3 X^{n-3} + \ldots + g_i X^{n-i} + \ldots + g_n, \end{aligned}$$

where the nonzero g_i are given by $g_i = t_2^{\sigma_i}\pi_i$; $\sigma_i = nr_i + r_n(n-1) - nr_n = nr_i - ir_n \geq 0$, by (II); and $\pi_i = (t_1 + d)^{r_i}\delta_i$ = a unit in $\mathfrak{S}^*$. In particular, $\sigma_n = nr_n - nr_n = 0$, and hence $h_n = \pi_n$ = a unit in $\mathfrak{S}^*$.

Let $k^* = \mathfrak{S}/\mathfrak{m}^*$, and let $\bar{G}(X) = X^n + \bar{g}_2 X^{n-2} + \ldots + \bar{g}_i X^{n-i} + \ldots + \bar{g}_n$, where $\bar{g}_i$ is the $\mathfrak{m}$ residue of g_i. Let $\bar{H}(X)$ be an irreducible factor of $\bar{G}(X)$ in $k^*[X]$. By Corollary 3 of Lemma 3, there exists a real discrete valuation v of K with center $\mathfrak{m}^*$ in $\mathfrak{S}^*$ such that $\bar{H}(X)$ has a linear factor $(X - l)$ in $(R_v/M_v)[X]$. If $(X - l)$ were the only irreducible factor of $\bar{G}(X)$ in $(R_v/M_v)[X]$, we would have $\bar{G}(X) = (X - l)^n$, and hence $l^n = \bar{g}_n \neq 0$ and $nl = \bar{g}_1 = 0$, which is a contradiction, since $n \not\equiv 0(p)$ and since R_v/M_v is of characteristic p. Therefore, $\bar{G}(X)$ has at least two relatively prime factors in $(R_v/M_v)[X]$, and hence by Hensel's Lemma (and by Theorem V on p. 303 of Ostrowski, *op. cit.*) we conclude that v has more than one extension to K^*. Again the infinitely many choices of the maximal ideal m in S give us infinitely many valuations of the required type.

2. *An Application to Galois Theory.*—Let (o, m) be an integrally closed local domain with quotient field K, and let K^* be a finite separable normal extension of K. Let G be the Galois group of K^*/K. Let o^* be the integral closure of o in K^*. Then o^* is a semilocal domain. Let m^* be a maximal ideal in o^*, and let G_s and K_s be, respectively, the splitting group and the splitting field of m^* over m. Let $\bar{o} = K_s \cap o^*$, $m = K_s \cap m^*$, $o_s = \bar{o}_{\bar{m}}$ and $m_s = \bar{m}o_s$. It was conjectured by Krull[7] and recently proved by Nagata[8] that $m_s = mo_s$. As an application of Theorem 1, we shall give a new proof of this equality under the following three assumptions: (I) The completion of o_s is a domain. This condition is certainly satisfied if either o_s or o is a geometric local ring, or more generally a local ring with a nucleus.[9] (II) o/m is a field of the same characteristic as K. (III) o is regular.

We need the following lemma:

LEMMA 4. *Let (o, m) be a local domain whose completion $(o, \bar{m})$ has no proper zero divisors. Let K and $\bar{K}$ be the respective quotient fields of o and $\bar{o}$. Let Ω be the set of all real valuations of K having center m in o, and let $\bar{\Omega}$ be the set of all real valuations of $\bar{K}$ having center $\bar{m}$ in $\bar{o}$. Let f be the map which takes a valuation in $\bar{\Omega}$ into its K-restriction. Then, up to the equivalence of valuations, f maps in one-to-one fashion $\bar{\Omega}$ onto a subset of Ω.*[10]

Proof: It is obvious that f maps $\bar{\Omega}$ into Ω. Also, for any two equivalent valuations v_1 and v_2 in $\bar{\Omega}$ it is obvious that $f(v_1)$ and $f(v_2)$ are equivalent. So the only thing that remains to be proved is that, for any v_1 and v_2 in $\bar{\Omega}$, if $f(v_1)$ and $f(v_2)$ are equivalent, then so are v_1 and v_2.

Let then v_1 and v_2 be two elements in $\bar{\Omega}$ such that $w_1 = f(v_1)$ and $w_2 = f(v_2)$ are are equivalent. Then there is a mapping τ which maps the value group Γ_1 of w_1 isomorphically onto the value group Γ_2 of w_2 such that $w_2(c) = \tau w_1(c)$ for all c in K. Let a be any given element of $\bar{o}$. Then we can find $a_i \in o$ such that $a - a_i \in \bar{m}^i$ for all i. Let $\{u_1, u_2, \ldots, u_s\}$ be a basis of $\bar{m}$. Let $t_1 = \min\{v_1(u_1), v_1(u_2), \ldots, v_1(u_s)\}$. Then, clearly, $v_1(y) \geq ht_1$ for all $y \in \bar{m}^h$. Let l_1 be an integer $> v_1(a)/t_1$. Since for any $l \geq l_1$ we have $v_1(a - a_l) \geq lt_1 \geq l_1t_1 > v_1(a)$, it follows that $w_1(a_l) = v_1(a_l) = v_1[a + (a - a_l)] = v_1(a)$. Similarly, there exists an integer l_2 such that for any $l \geq l_2$ we have $w_2(a_l) = v_2(a)$. Let $l = \max(l_1, l_2)$. Then $v_2(a) = w_2(a_l) = \tau w_1(a_l) = \tau v_1(a)$. Furthermore, given any c in $\bar{K}$, we can write $c = a/b$ with $a, b \in \bar{o}$; and then $v_2(c) = v_2(a) - v_2(b) = \tau v_1(a) - \tau v_1(b) = \tau[v_1(a) - v_1(b)] = \tau v_1(c)$. Thus Γ_1 and Γ_2 are the value groups of v_1 and v_2, and τ maps Γ_1 isomorphically onto Γ_2 such that $v_2(c) = \tau v_1(c)$ for all c in $\bar{K}$; i.e., v_1 and v_2 are equivalent.

THEOREM 2. *The equality $m_s = mo_s$ holds under assumptions I, II, and III.*

Proof: If $\dim o = 1$, then o is the valuation ring of a real discrete valuation of K. In this case the theorem is well known.[11] Now assume that $\dim o > 1$. Let v be any valuation of K with center m in o. Let $v_1^*, v_2^*, \ldots, v_l^*$ be those extensions of v to K^* which have center m^* in o^*. It is then clear that the elements of G_s permute the elements $v_1^*, v_2^*, \ldots, v_l^*$ among themselves and that, if an element τ of G sends one element of the set $\{v_1^*, v_2^*, \ldots, v_l^*\}$ into an element of the same set, then τ belongs to G_s. In other words, if G is thought of as a group of permutations of the extensions of v to K^*, then $\{v_1^*, v_2^*, \ldots, v_l^*\}$ is a set of imprimitivity of that group. Since K_s is the fixed field of G_s and since any two extensions of v to K^* are conjugate to each other over K,[11] it follows that $v_1^*, v_2^*, \ldots, v_l^*$ have a common contraction to K_s, say v_s, that $v_1^*, v_2^*, \ldots, v_l^*$ are the only extensions of v_s to K^* and that v_s is the only extension of v to K_s which has center m_s in o_s. Thus we have shown that any valuation of K with center m in o has only one extension to K_s having center m_s in o_s.

Let $(\bar{o}_s, \bar{m}_s)$ and $(\bar{o}, \bar{m})$ be the respective completions of o_s and o. Let Σ_s and Σ be the respective quotient fields of $\bar{o}_s$ and $\bar{o}$. In a canonical way we can consider Σ to be a subfield of Σ_s. Σ_s is then a finite separable extension of Σ.[12] Suppose, if possible, that $\Sigma_s \neq \Sigma$. Then, by Theorem 1, there exists a real valuation $\bar{v}$ of Σ with center $\bar{m}$ in $\bar{o}$, which has at least two distinct extensions $\bar{v}_s^{(1)}$ and $\bar{v}_s^{(2)}$ to Σ_s. Obviously $\bar{v}_s^{(1)}$ and $\bar{v}_s^{(2)}$ have center $\bar{m}_s$ in $\bar{o}_s$. Let v be the restriction of $\bar{v}$ to K, and let $v_s^{(1)}, v_s^{(2)}$ be the respective restrictions of $\bar{v}_s^{(1)}, \bar{v}_s^{(2)}$ to K_s. Then v has center m in

o, and $v_s^{(1)}$ and $v_s^{(2)}$ have center m_s in o_s. Since, by Lemma 4, $v_s^{(1)} \not\equiv v_s^{(2)}$ (i.e., non-equivalent), this is a contradiction.

Therefore, $\Sigma_s = \Sigma$, and hence $\bar{o}_s = \bar{o}$, $\bar{m}_s = \bar{m}$. Since $\bar{m}\bar{o} = \bar{m}$ and $\bar{m}_s \cap o_s = m_s$, it follows that $m_s = mo_s$.

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[1] See notations below.

[2] This proof follows closely Zariski's proof of Theorem 4 in "Foundations of a General Theory of Birational Correspondences," *Trans. Am. Math. Soc.*, **53**, 490–542, 1943.

[3] W. Krull, *Idealtheorie* (Berlin, 1935), p. 108.

[4] This proof is based on considerations in sec. 4 of O. Zariski, "A Simplified Proof for the Resolution of Singularities of an Algebraic Surface," *Annals*, **43**, 583–593, 1942. See also sec. 11 of Zariski, *Trans. Am. Math. Soc.*, **53**, 490–542, 1943.

[5] Zariski, *Trans. Am. Math. Soc.*, **53**, 490–542, 1943.

[6] A. Ostrowski, "Untersuchungen zur arithmetischen Theorie der Körper," *Math. Z.*, **39**, 269–404, 1934; see p. 298, Theorem III, and p. 303, Theorem V.

[7] W. Krull, "Galoissche Theorie der ganz abgeschlossenen Stellenringe," *Sitzber. Physik.-med. Sozietät Erlangen*, **67/68**, 324–328, 1936, Theorem 2.

[8] M. Nagata, "On the Theory of Hensilian Rings," *Nagoya Math. J.*, **5**, 45–57, 1953, Theorem 1.

[9] P. Samuel, *Algèbre locale* (Paris, 1953), pp. 37–39, 57–58.

[10] I.e., for any v_1 and v_2 in Ω we have: $f(v_1)$ and $f(v_2)$ are equivalent if and only if v_1 and v_2 are equivalent.

[11] M. Deuring, "Verzweigungstheorie bewerteter Körper," *Math. Ann.*, **105**, 277–307, 1931.

[12] C. Chevalley, "On the Theory of Local Rings," *Annals of Math.*, **44**, 690–708, 1943.

INVARIANTS D'UNE APPLICATION CONTINUE

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1. Dans une conférence au Congrès International de 1950,[1] E. H. Spanier, utilisant une méthode qu'il avait introduite avec S. S. Chern pour l'étude des espaces fibrés en sphères,[2] définit pour un espace fibré X, dont la base Y est un complexe fini de dimension n, le diagramme suivant:

$$\begin{array}{ccccccc}
 & & & & H^p(X_n = X; G) & \xleftarrow{j^*} & H^p(X_n, X_{n-1}; G) \longleftarrow \cdots \\
 & & & & \downarrow{\scriptstyle i^*} & & \\
\cdots \longleftarrow H^{p+1}(X_n, X_{n-1}; G) & \xleftarrow{\delta^*} & H^p(X_{n-1}; G) & \xleftarrow{j^*} & H^p(X_{n-1}, X_{n-2}; G) & & \cdots \\
 & & \downarrow{\scriptstyle i^*} & & & & \\
\cdots \longleftarrow H^{p+1}(X_{n-1}, X_{n-2}; G) & \xleftarrow{\delta^*} & H^p(X_{n-2}; G) & \longleftarrow & \cdots\cdots & & \\
 & & \downarrow & & & & \\
 & & \cdots\cdots & & \cdots\cdots & & \cdots\cdots
\end{array}$$

où, ξ étant la projection de X sur sa base Y, et Y_m le m-squelette de Y, on a $X_m = \xi^{-1}Y_m$. G est l'image par ξ^{-1} d'un système de groupes locaux sur Y.

Spanier indiqua que ce diagramme est équivalent à la suite spectrale de l'espace fibré X,[3] et remarqua aussi que l'on obtient un diagramme semblable en considérant

les groupes d'homotopie relatifs des squelettes d'un complexe. Ces idées ont été reprises dans les mêmes hypothèses par W. S. Massey qui appelle le diagramme de Spanier un couple exact et développe les calculs correspondants.[4]

Nous nous proposons dans cette note de définir un diagramme du même type que celui de Spanier, dans la situation très générale d'une application continue quelconque ξ d'un espace topologique X dans un espace topologique Y. Du diagramme obtenu, on peut déduire la suite spectrale de ξ.[5] De plus, il s'introduit de nouveaux invariants d'une application continue ξ; ceux-ci sont de type fini lorsque ξ est de type fini (par exemple lorsque ξ est fibrée et que la base Y et la fibre F sont de types finis).

2. Soit A un anneau possédant deux degrés d_0, $d_1 \geq 0$ compatibles ($A = \sum_{p,q} A^{p,q}$; si $a^{p,q} \in A^{p,q}$, $d_0(a^{p,q}) = q$, $d_1(a^{p,q}) = p$), et deux différentielles δ_0, δ_1, associées respectivement aux degrés d_0, d_1 (δ_i de degré $+1$ relativement à d_i, $i = 0, 1$), compatibles: $\delta_0\delta_1 = \delta_1\delta_0$.

Ces deux structures sur A déterminent une structure totale dont le degré est $d = d_0 + d_1$ ($^nA = \sum_{p+q=n} A^{p,q}$), et la différentielle $\delta = \delta_1 + \beta\delta_0$, β étant l'automorphisme de A: $\beta a^{p,q} = (-1)^p a^{p,q}$.[6]

z_i, δ_i, h_i et z, δ, h désigneront respectivement les cycles, les bords et l'homologie relativement à δ_i, $i = 0, 1$ et à δ. Une relation fondamentale entre l'homologie des structures partielles et de la structure totale est fournie par la suite spectrale obtenue en filtrant[3] A par $A_s = \sum_{p \geq s} A^{p,q}$. Son terme $E_2 = h_1h_0A$. La construction suivante donne une analyse des différentielles δ_r de la suite spectrale précédente en fonction des structures partielles. Partons des suites exactes:

$$0 \to z_0A \to A \to \delta_0A \to 0,$$

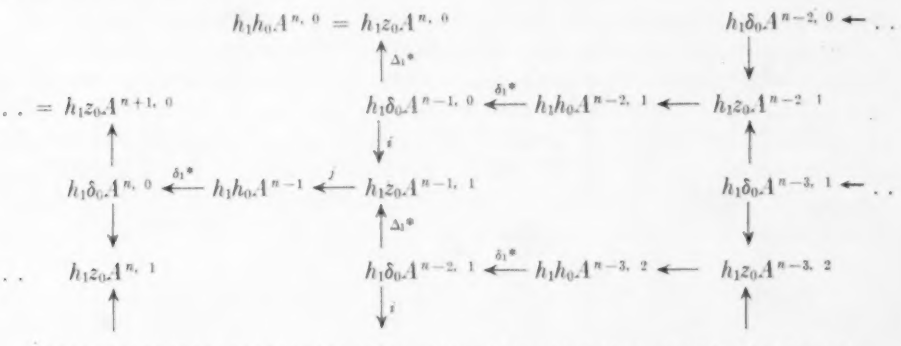
$$0 \to \delta_0A \to z_0A \to h_0A \to 0.$$

δ_1 induit une différentielle sur z_0A, δ_0A, h_0A, et détermine pour chaque entier $q \geq 0$ les deux suites exactes:

$$0 \to h_1z_0A^{0,q} \to h_1A^{0,q} \to h_1\delta_0A^{0,q} \xrightarrow{\Delta_1^*} h_1z_0A^{1,q} \to \ldots, \qquad (1)_q$$

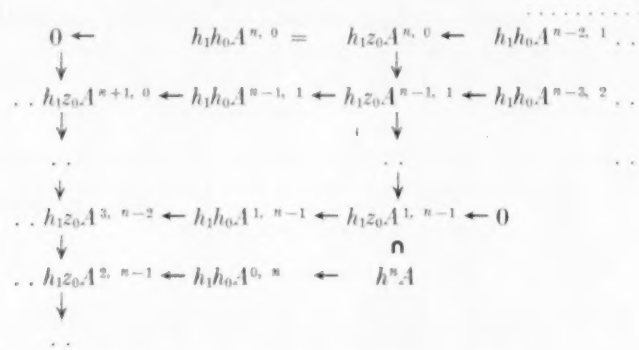
$$0 \to h_1\delta_0A^{0,q} \to h_1z_0A^{0,q} \to h_1h_0A^{0,q} \to h_1\delta_0A^{1,q} \to \ldots, \qquad (2)_q$$

d'où l'on tire le tableau



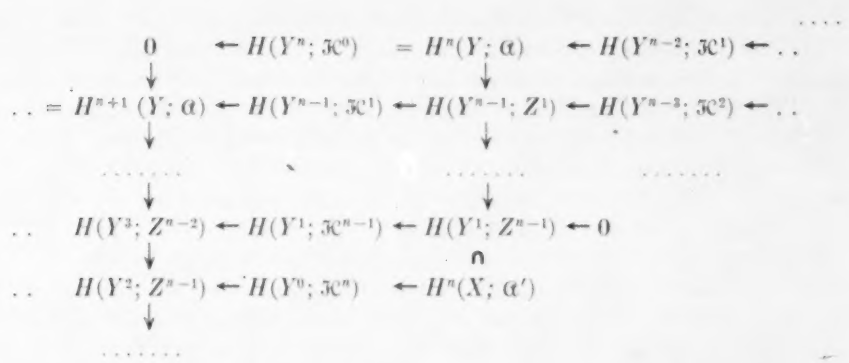
$j \circ \Delta_1^* \circ \delta_1^*$, qui applique $E_2^{p,q} = h_1 h_0 A^{p,q}$ dans $E_2^{p+2,q-1}$, est la différentielle δ_2 de la suite spectrale. $j \circ \Delta_1^* \circ i^{-1} \circ \Delta_1^* \circ \delta_1^*$ engendre la différentielle δ_3, déterminant un homomorphisme du sous-anneau des δ_2-cycles de $E_2^{p,q}$ dans le quotient des δ_2-cycles de $E_2^{p+3,q-1}$ par l'image $\delta_2 E_2^{p+1,q}$, et annulant $\delta_2 E_2^{p-2,q+1} \subset E_2^{p,q}$. D'une maniere analogue, $j \circ \Delta_1^* \circ i^{-1} \circ \ldots i^{-1} \circ \Delta_1^* \circ \delta_1^*$ où entrent $r-1$ homomorphismes Δ_1^* alternant avec $r-2$ i^{-1}, $r \geq 2$, détermine δ_r. Dans les applications, la situation est souvent simplifiée par la relation suivante: $h_1 A^{p,q} = 0$ si $p > 0$, pour chaque $q \geq 0$. Elle a pour conséquences (*a*) Δ_1^*: $h_1 \delta_0 A^{p,q} \to h_1 z_0 A^{p+1,q}$ est un isomorphisme sur si $p > 0$, un homomorphisme sur si $p = 0$ (d'après $(1)_q$); (*b*) $h^n A = h\left(\sum_{p+q=n} A^{p,q}\right) = h_0 h_1 A^{0,n}$ (en vertu d'un raisonnement direct ou de la suite spectrale relative à la filtration de A par d_0). Or: $h_0 h_1 A^{0,n} = h_0 z_1 A^{0,n} = z_0 z_1 A^{0,n}/\delta_0 z_1 A^{0,n-1} = h_1 z_0 A^{0,n}/\delta_0 h_1 A^{0,n}$. D'autre part, le début de la suite exacte $(1)_n$ se transforme en remarquant que $h_1 A^{0,n}/h_1 z_0 A^{0,n} = z_1 A^{0,n}/z_1 z_0 A^{0,n} = \delta_0 z_1 A^{0,n} = \delta_0 h_1 A^{0,n}$, en $0 \to \delta_0 h_1 A^{0,n} \to h_1 \delta_0 A^{1,n} \to h_1 z_0 A^{1,n} \to 0$.

On peut donc remplacer $h_1 z_0 A^{1,n-1} \xleftarrow[(sur)]{\Delta_1^*} h_1 \delta_0 A^{0,n-1} \subset h_1 z_0 A^{0,n}$ par $h_1 z_0 A^{1,n-1} \subset h^n A$, et l'on obtient le diagramme simplifié:



3. Soit ξ une application continue de l'espace X dans l'espace Y. Si W est un ouvert de X, $(W; \mathfrak{a}')$ désignera l'anneau des cochaines singulières ou d'Alexander-Čech de W, à coefficients dans le faisceau $\mathfrak{a}'$, image inverse par ξ^{-1} d'un faisceau localement constant $\mathfrak{a}$ sur Y. Si V est un ouvert de Y, soit $B(V) = (\xi^{-1}V; \mathfrak{a}')$. Les $B(V)$ forment un faisceau B d'anneaux différentiels gradués sur Y. Soit $A = (Y; B)$, l'anneau des cochaines d'Alexander-Čech de Y à coefficients dans B.

L'anneau A possède deux structures différentielles graduées compatibles. Si Z, $\mathfrak{H}$ désignent les faisceaux des cocycles, resp. de cohomologie du faisceau B, les termes $h_1 h_0 A^{p,q} = H(Y^p; \mathfrak{H}^q)$ sont les termes $E_2^{p,q}$ de la suite spectrale de ξ. Les termes $h_1 z_0 A^{p,q} = h_1 z_0 (Y^p; B^q) = H(Y^p; Z^q)$ sont de nouveaux invariants de ξ. On a de plus $h_1 z_0 A^{n,0} = H(Y^n; Z^0) = H^n(Y; \mathfrak{a})$ et $h^n A = h_0 h_1 A^{0,n} = h_0 h_1 (Y^0; B^n) = h_0 B^n(Y) = h_0(X^n; \mathfrak{a}') = H^n(X; \mathfrak{a}')$, d'où le diagramme



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*PROOF FOR AN ENDOGENOUS COMPONENT IN PERSISTENT SOLAR AND LUNAR RHYTHMICITY IN ORGANISMS**

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A question often raised but never answered is that of the site of control of persistent diurnal rhythms. Even a brief consideration of the data available concerncerning the diurnal rhythm of color change leads to the conclusion that the phase relationships between the diurnal rhythms as exhibited by animals and that of the normal environment are subject to alteration by appropriate methods, and the rhythm then persists in the new phase relationships as long as the animals are maintained in constant darkness. Thus the diurnal rhythm can be reversed by reversed illumination; subsequently, in constant darkness the reversed rhythm persists.[1] Similarly, by a 6-hour period of illumination (1 A.M. to 7 A.M.), a shift of 6

hours in the time of occurrence of each phase can be obtained, and this also persists when the animals are thereafter maintained in constant darkness.[2] It is also possible to alter the phase relationships by exposing the animals to low temperature and then returning them to room temperature.[3, 4] By this method any phase of the diurnal rhythm of color change can be made to occur at any time of day, and upon return to room temperature in darkness the rhythm persists in its new phase relations. It appears, then, that any one of several physical factors in the normal environment can set the phase relations.

There have, however, been no experiments performed to learn to what extent the precise 24-hour cycles of the daily, and the 24.8-hour cycles of the lunar, rhythm could continue to be maintained when all corresponding external factors were rendered nonrhythmic or with other than normal frequency. The following experiment was, therefore, designed to determine whether the usual daily and lunar cycles of the fiddler crab could be maintained during a time when the physical cycles were artifically attenuated by rapid east-to-west travel by airplane. Could the animals measure off cycles of the normal length during this period when no external cues could be available, and, if so, with what probable accuracy? And if one cycle were to be measured with accuracy during the day of the trip, would the phase relationships thereafter in the new locality be permanently shifted with respect to the local solar and lunar time as in the cases of light- or temperature-treated animals? An alternative could be that the crabs which were shown capable of maintaining 24-hour cycles even when out of normal phase relationships with solar day-night gradually would drift under conditions of constant light and temperature until their phases with respect to some external physical cycles came to bear the same relations as had originally been determined by light and tides in their former habitat. In other words, would the clocks of the crabs in California gradually drift under these conditions to having their phases about $3^1/_2$ hours later than those of the Woods Hole crabs?

MATERIALS AND METHODS

On August 17, 1954, at 1 P.M. two groups of 250 animals each were placed in two wooden buckets, together with enough moist *Fucus* to fill each bucket about two-thirds full. The cover of each bucket had several holes bored through it, and to the undersurface of each cover was secured a black cloth. The covers were held on by nails. One such bucket of crabs was kept in the laboratory at the Marine Biological Laboratory, Woods Hole, Massachusetts, while the other was transported by car to Logan Airport, Boston, Massachusetts. From there it was carried in the cabin of a commercial airlines plane to San Francisco. The take-off time from Logan was 5:40 P.M. Eastern Standard Time; the arrival time at International Airport, San Francisco, was 4:00 A.M. Pacific Standard Time. Brief stops were made en route at Hartford-Springfield and at Chicago. Upon arrival at San Francisco the bucket was taken by automobile to the laboratories of the Department of Zoölogy, University of California, at Berkeley. There the bucket was opened at 10 A.M. (PST) and the animals separated into three groups, each of which was placed in a white enameled container with a small amount of sea water. The containers with the animals were placed in a room in which the illumination was maintained constant at an intensity of less than 1 ft-c. (estimated). At the same time (i.e., 1

P.M. EST) the animals in Woods Hole were removed from the bucket, placed in comparable white enameled containers, and exposed to a constant illumination, similarly of less than 1 ft-c.

Beginning at 1 P.M. PST (4 P.M. EST) on August 18, the state of the chromatophores was determined for the crabs in the two geographical sites at hourly intervals until the time when the pigment was clearly in the night phase. Subsequent simultaneous determinations at the two locations were made each morning as the pigment was in transition from night-to-day phase and each afternoon and evening as it was moving from daytime-to-nighttime state. In general, determinations were made at 4, 5, 6, 7, 8, 9, 10, 11, and 12 P.M. and at 4, 5, 6, 7, 8, 9, and 10 A.M. EST and at the corresponding hours PST, at 1, 2, 3, 4, 5, 6, 7, 8 and 9 P.M. and at 1, 2, 3, 4, 5, 6, and 7 A.M. PST. The number of determinations and the hours at which they were made can be seen in Figure 1, in which each point on the curve represents the average obtained from assaying 50 animals. The last determinations were made at 10 A.M. EST on August 24 (7 A.M. PST).

RESULTS

The results are graphically represented in Figure 1, in which the average state of the chromatophores is plotted as a function of time of day. Figure 1 shows the state of the chromatophores of the animals in Woods Hole at the times observed and the simultaneous condition for the animals being observed at Berkeley.

It can be seen that at no time are there any great differences, in general, between the two curves. Thus the animals being observed in Berkeley entered the night phase at approximately the same time as did the animals being observed in Woods Hole, and similarly the two groups of animals entered the day phase almost simultaneously. Small differences were observed on several days. These differences are summarized in Table 1, in which are compared the times at which the chromatophores of the two groups of animals passed through stage 2.5. This stage was arbitrarily selected for this comparison, inasmuch as this always occupied a position of rapid transition between the day and night phases, and hence any temporal difference in the phases of the cycles would be most sharply delineated here.

TABLE 1

TIMES OF 2.5 VALUES

DATE	WOODS HOLE, MASSACHUSETTS (EST) A.M.	P.M.	BERKELEY, CALIFORNIA (EST) A.M.	P.M.
Wednesday, Aug. 18	..	7:00	..	7:10
Thursday, Aug. 19	6:40	7:45	6:40	8:20
Friday, Aug. 20	5:30	8:15	6:30	9:15
Saturday, Aug. 21	7:05	9:30	6:20	10:10
Sunday, Aug. 22	6:15	10:10	6:50	...
Monday, Aug. 23	7:20	10:30	7:15	10:45
Tuesday, Aug. 24	7:30	...	8:00	...
Average	6:44	8:36	6:56	9:08 (av. of 5 values)

At Woods Hole the chromatophores passed through stage 2.5 at the average time for the six days of 6:44 A.M. EST, while at California they were passing through this stage at 6:56 A.M. EST. In the evening, stage 2.5 was passed at the average time for the observation period of 8:36 P.M. EST for the (Woods Hole animals and 9:08 P.M. EST (average of 5 values) for the Berkeley ones.

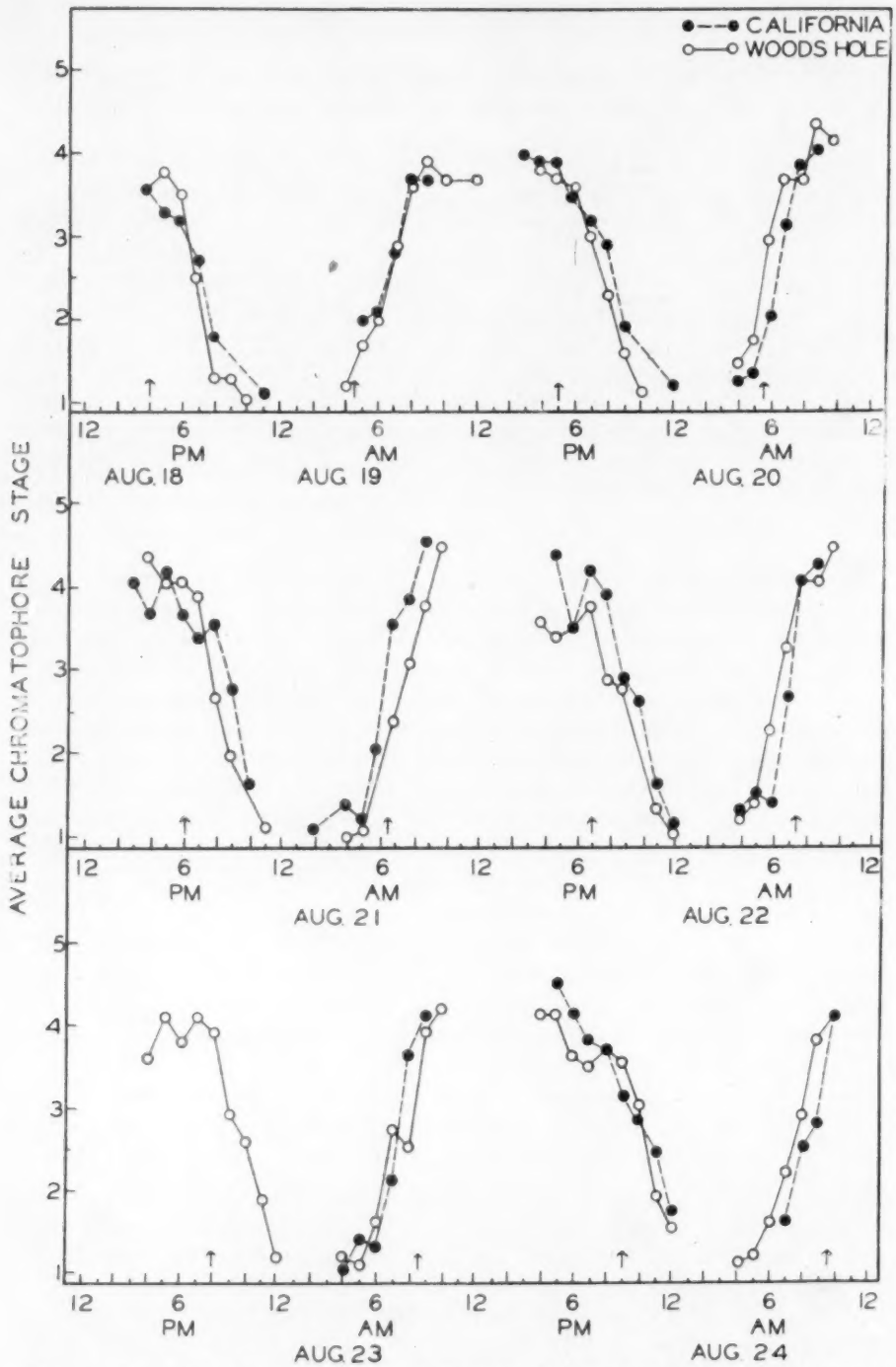

FIG. 1.—The cycles of change in degree of dispersion of the melanin in the melanophores of *Uca pugnax* in Berkeley, California (122° W.; 38° N.), compared with similar cycles for control animals kept in Woods Hole, Massachusetts (71° W.; 42° N.). The cycles were determined under similar constant conditions. All the times are Eastern Standard. The arrows indicate the times of low tide in the place of collection of the crabs.

Table 1 shows that, for the six days during which observations were made, the time of entering the day phase was, on the average, 12 minutes later for the animals in Berkeley than for those at Woods Hole. The time of entering the night phase was found to average 32 minutes later for the crabs in Berkeley than for those in Woods Hole. The average transition point for the two times of day in Berkeley was then 22 minutes later than in Woods Hole.

Further study of the data indicates that there is a general drift of both the morning and evening times of occurrence of stage 2.5. This is evident, for example, in comparing the average of the evening times for the dates August 18–20 with those of August 21 and 23. For the dates August 18–20 the Woods Hole crabs average 56 minutes *earlier* than their mean for the six days, and the California crabs average 53 minutes *earlier* than theirs. In contrast, for the period from August 21 through August 23 the two groups are respectively 84 and 80 minutes *later* than their six-day means.

A similar situation is seen for the times of the morning 2.5 stage. For the first three mornings of observation the Woods Hole crabs averaged 19 minutes *earlier* than their six-day mean, and the Berkeley ones averaged 26 minutes *earlier* than their six-day mean. For the last three days of observation, August 22 through 24, the times were, respectively, 14 and 26 minutes *later* in the day for the two groups of animals.

Now, considering the average values obtained for both morning and evening, the Woods Hole and California crabs averaged 38 and 40 minutes *earlier* than their mean during the first three days, and 49 and 53 minutes *later*, respectively, than their means for the last three days.

If one examines Figure 1, it is clearly seen that on all five evenings the Pacific Coast crabs lighten later than the Woods Hole ones. For the morning hours, three out of six values show the California crabs to darken later than the Woods Hole ones, and in only one instance was darkening earlier.

DISCUSSION

In view of the fact that it seems unlikely that two randomly picked groups of 250 crabs taken from the same large sample would exhibit, when sampled in lots of 50 at hourly intervals, an average difference of 22 minutes in phase relations of their color-change rhythm, and that this average difference did not become altered between the first half and the second half of the six-day period during which they were simultaneously studied in the two geographical sites, it seems probable that the 22-minute difference came about during the 24-hour period intervening between their being packed at Woods Hole and their being simultaneously unpacked at the two sites.

The alternative possibility of the initial samples differing by that amount could have occurred, however, even though it seems less probable in view of the equal conciseness of the daily rhythms for the two samples and the remarkably parallel character of the transitions between day and night phases. The steep slopes of transition suggest relatively homogeneous populations of crabs with respect to these temporal differences.

Since the successive points on the curves describing the chromatophore changes were obtained by reading successive groups of crabs of a three-pan series, the consistency of the trends argues against an error of sampling of the groups of 50 assayed

each hour alone being responsible for the differences between the samples in the two sites or for the day-by-day variations in any site. What the factors are which are responsible for the latter are still not known.

The general drift of the times of the morning and afternoon 2.5-values, to the extent that the values for the last three days average 87 minutes later in the day than the first three for the Woods Hole crabs and 93 minutes later for the Berkeley ones, is at least in part explicable in terms of passage of the primary lunar or tidal cycle over the diurnal. It can be seen by the positions of the arrows in Figure 1 that the times of low tide occur at a series of times during the six-day period which would be expected to move both A.M. and P.M. transitional values gradually later in the day.[5] It seems very significant that the tidal rhythm of the Berkeley animals apparently had the same relationship to the diurnal as did that of the Woods Hole ones. It is quite obvious that, if one assumes that the daily cycle has not actually been altered in one sample of crabs and calculates the drift of the tidal influence on the basis of the average daily cycle of the Woods Hole and Berkeley crabs, it will appear that either the tidal was abruptly shifted forward about 20 minutes en-route to California or the two original samples possessed this difference in tidal phase relationships initially.

It is quite clear that, if one ignores ordinary temperature fluctuations and the barometric pressure and correlated geophysical and cosmic changes experienced during transport, the crabs in California were, like the crabs in Woods Hole, in a physical environment where a number of known forces possess cycles correlated in frequency with the periods of rotation of the earth relative to sun and moon. Only during the 12 hours in transit was the experimental situation different from ones obtainable in either site alone. During the day including the period of transit, all those rhythmic changes in forces effected by rotation of the earth relative to the sun had one cycle, relative to the animals, extended from 24 hours to about 27.33 hours, and all those changes relative to the moon altered from a 24.8-hour period to one of about 28.1 hours. During the day on which the crabs were being transported westward, they could not conceivably have been receiving any external signal, which would have recurred at 24 hours or 24.8 hours to have permitted either the daily or the lunar period to have been measured so accurately by the organism.

Although the variation in the values from day to day was so great that the exact extent of the change of the phases of the rhythm cannot be ascertained, the data strongly suggest that it approximated 20 minutes. If one relies not only on the differences between the 11 available transitional 2.5-points but utilizes 33 transitional points, namely those at 2.0, 2.5, and 3.0, one obtains a mean difference of $+25.2 \pm 6.26$ minutes. With the average difference 4.02 times the standard error of the mean, there would be only 1 chance in more than 15,000 that the measured average difference in phase between the crabs in the two sites was fortuitous. The difference is, therefore, quite significant. Furthermore, there is about a 20 to 1 chance that the true mean lies within the range of twice the standard error, or between $+12.7$ and $+37.7$ minutes. The interpretation of the difference between the two groups, i.e., whether due to inaccuracy of an endogenous clock, a shift induced by some cosmic factor or factors during the single attenuated solar and lunar day, or some small difference in the illumination changes as the crabs were packed or un-

packed, is obviously a matter which is yet to be resolved. But this statistical analysis also renders it even more obvious that the crabs did not follow the change in solar time resulting from the east-west transport.

The problem of the maintenance of the same average frequency, but now with the phases of the cycle of color change about 3 hours different relative to the day-light-dark change in the two sites, offers again the same problem as in the precise frequency maintenance of rhythms of color change with phase relations abruptly shifted relative to the external physical cycles by light or low temperature. This research offers no solution to this problem, in which the mechanism involved does not permit drift significantly out of phase with the solar day-night cycle even over months (e.g., Welsh,[6] Brown and Webb,[3] and Webb[2]).

Irrespective of the exact degree of precision of the solar and lunar cycles of color change in animals out of their normally rhythmic environment as during east-west transport, the results clearly indicate the possession of a reasonably accurate biological clock. Whether or not this clock normally requires continuous resetting by some daily and lunar cycles of the external physical world, such a biological clock would seem theoretically to be a necessary component in the mechanism of the normally precise rhythms of solar and lunar frequency occurring under constant conditions. In the event that the biological clock is only moderately accurate and normally needs continuous resetting, as is suggested by these results, use of the rhythmic character and phase relationships of the physical cycles would probably require the integration by the organism of several consecutive days of stimuli in order to render evident the rhythmic components in the midst of the ordinarily large random variations.

SUMMARY

1. By transportation of fiddler crabs 51° westward (from Woods Hole, Massachusetts, to Berkeley, California) within a 24-hour daily period, it was possible to determine the approximate degree of precision of the capacity of the animals to maintain 24-hour daily and 24.8-hour lunar cycles of color change while no concurrent physical cycle of the same length could be affecting the animals.

2. Compared with control animals kept in Woods Hole, the Berkeley crabs during six days of observation exhibited cycles shifted to a slightly later time in the day, the average shift for eleven morning and evening transitional points being 22 minutes forward.

3. When the results from the first three days in both Berkeley and Woods Hole were compared with those from the last three days, there appeared to be no tendency for any drift in the cycles of the California crabs away from the controls in Woods Hole during this period, the animals, therefore, averaging relatively precise 24-hour cycles during the six-day period in each of the two sites.

4. The variations in the form of the daily cycles, owing at least in part to the possession of a simultaneous persistent tidal rhythm, continued to bear the same temporal relationships to the daily in the Berkeley crabs that they bore to the daily in the Woods Hole ones.

5. It seems clear, therefore, that, devoid of all possible rhythmic external signals, the crabs are able to mark off with quite good accuracy periods of solar and lunar day lengths.

6. Reasons are advanced for the necessity for a reasonably accurate biological component in any mechanism regulating solar and lunar rhythmicity under conditions of constant illumination and temperature.

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